THESIS

THE EFFECT OF LIPID AND OXYGEN CONCENTRATION ON MYOSIN HEAVY CHAIN ISOFORM EXPRESSION IN C2C12 AND CULTURED WEDDELL SEAL (*LEPTONYCHOTES WEDDELLII*) SKELETAL MUSCLE CELLS

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ABSTRACT

THE EFFECT OF LIPID AND OXYGEN CONCENTRATION ON MYOSIN HEAVY CHAIN ISOFORM EXPRESSION IN C2C12 AND CULTURED WEDDELL SEAL (*LEPTONYCHOTES WEDDELLII*) SKELETAL MUSCLE CELLS

The Weddell seal (*Leptonychotes weddellii*), a diving mammal, has large stores of myoglobin as compared to terrestrial mammals. Understanding the regulation of myoglobin in this unique species has therapeutic potential in people at risk of muscle infarction; to pursue this end, we established a successful protocol for the isolation and culture of primary Weddell seal skeletal muscle cells utilizing lipid-supplemented culture media. Using these cells, a study was designed to determine if increasing lipid in the media in either normoxic or hypoxic conditions would affect the expression of myosin heavy chain (MHC), which might explain a previously observed increase in myoglobin expression with increased lipid. Gel electrophoresis consistently resolved two putative MHC isoforms from seal and c2c12 cells. LC-MS/MS determined that the resolved bands were heterogeneous, and that all bands contained minimal adult MHC (10% or less, as measured by NSAF). Despite the minimal expression of adult MHC isoforms, myoglobin was detected in each treatment group. This study suggests that electrophoresis methods alone may not be sufficient to detect changes in MHC isoform abundance in cell culture, and that, in cell culture, regulatory pathways may exist for myoglobin that are independent of adult fiber type.

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CHAPTER 1 – INTRODUCTION

The Weddell seal (*Leptonychotes weddellii*) utilizes aerobic metabolic pathways during breath-hold exercise while diving (Kooyman et al., 1980; Williams et al., 2000). One of the adaptations facilitating the use of aerobic pathways is the relatively high concentration of myoglobin, an oxygen storage molecule, in its primary swimming muscle (*longissimus dorsi m*); the Weddell seal has 33 times more myoglobin in its swimming muscle than the *vastus medialis m* of a cotton rat (*Sigmodon hispidus*) (Kanatous et al., 1999; Kanatous et al., 2008). To further study the development of myoglobin regulation in a diving mammal, we developed a protocol to isolate and culture Weddell seal primary skeletal muscle cells. The successful culture of these cells required the addition of lipid to the proliferation and differentiation media, a supplement that is reflective of their diet; the development of this protocol is detailed in chapter 3 of this thesis.

Immortalized murine skeletal muscle cells (c2c12, ATCC® CRL-1772™) serve as terrestrial comparisons against Weddell seal (*Leptonychotes weddellii)* skeletal muscle cells during analyses of myoglobin concentration under different lipid and oxygen regimes (De Miranda et al. 2012). Myoglobin concentration in both mouse and seal muscle cells is known to change with transitions in skeletal muscle fiber type, and in response to an hypoxic environment coupled with a secondary stimulus, such as contractile stimulation during differentiation (Kanatous, 2009; *reviewed in* Kanatous and Mammen, 2010). Recently, De Miranda et al. (2012) showed that increasing the amount of lipid in the growth and differentiation media of Weddell seal skeletal muscle cells under normoxic conditions causes an increase in myoglobin

concentration. Additionally, within lipid treatment groups, myoglobin was greater in the cells subject to hypoxia. It is unknown whether or not the change with increasing lipid under normoxic conditions was due to a change in fiber type, as neither Weddell seal skeletal muscle cells grown in culture nor the c2c12 cell line have had their fiber type studied under varying lipid and oxygen conditions. While some studies have concluded that the fiber type of cultured primary myotubes from varying species is dependent on the fiber type of the muscle of origin, other studies suggest that fiber type in cell culture may be influenced by environmental conditions (Dusterhoff and Pette, 1993; Roseblatt et al., 1994; LaFramboise et al., 2003), and some have determined that clones of primary satellite cells can change their fiber type after multiple passages (Feldman and Stockdale, 1990; Ghosh and Doot, 1998). The fiber type of c2c12 myotubes has not been consistently described across different experimental conditions, suggesting an environmental component to fiber type determination (Allen et al., 2001; LaFramboise et al., 2003; Zebedin et al., 2004; Yuan et al., 2011). Due to possible environmental influence on fiber type, and the influence of fiber type on myoglobin expression, proper comparison of myoglobin regulation in mouse and seal skeletal muscle cell models under varying environmental conditions requires an understanding of how these varying conditions may affect fiber type.

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CHAPTER 2 – CHARACTERIZATION OF MYOSIN HEAVY CHAIN ISOFORMS IN CULTURED C2C12 AND WEDDELL SEAL (*LEPTONYCHOTES WEDDELLII*) SKELETAL MUSCLE CELLS

Introduction

De Miranda et al. (2012) found that increasing lipid concentration in the growth and differentiation medias of cultured Weddell seal skeletal muscle cells in normoxic conditions significantly increased myoglobin expression. This result was novel in that previous studies had described myoglobin regulatory pathways to be associated with either MHC isoform expression, or with exposure to hypoxia and a second stimulus; no lipid-dependent pathway had yet been characterized. To investigate the possibility that this increase in myoglobin was due to a lipid-induced fiber type change in the cells, this study was designed to characterize the MHC isoforms in cultured Weddell seal and c2c12 skeletal muscle cells in varying lipid and oxygen conditions.

Defining fiber type

Many organizational schemes have been developed for skeletal muscle fibers, and, while a standardized classification system has not materialized, fibers are commonly broadly categorized as having a "fast" or "slow" rate of contraction (Pearson, 1989). This categorization can be further refined by specific physiological and metabolic phenotypes, and motor neuron properties. Baraney (1967) determined that the activity of actin and Ca^{++} myosin ATPase, an enzyme component of myosin integral to the ratcheting mechanism in muscle contraction,

correlated with the speed of muscle shortening. Pette and Heilman (1979) determined that muscles composed mostly of fast twitch fibers had 10-fold higher activity of sarcoplasmic Ca^{++} ATPase, as well as higher Ca^{++} uptake than muscles composed mostly of slow twitch fibers, though they concluded that the transport protein was probably the same, and what varies are probably the amounts of the transporter, as well as other associated proteins. Peter et al. (1972) studied the muscles of guinea pigs and rabbits, and devised a classification scheme based on contraction times and metabolic phenotype, as defined by ATPase staining and enzymatic profile, respectively. Fibers deemed "fast twitch glycolytic" muscles had high ATPase staining intensity and high anaerobic enzyme activity; "fast twitch oxidative glycolytic" had intermediate to high ATPase staining intensity, the highest activity of the aerobic enzymes succinate dehydrogenase and cytochrome c, as well as the highest myoglobin concentration, and also a moderate to high glycolytic activity; lastly, "slow twitch oxidative" had low ATPase staining intensity, low anaerobic enzyme activity, and aerobic enzyme activity that was in between the other two classifications (Peter et al. 1972). Eccles et al. (1958) found that motor neurons innervating slow muscles have longer after-hyperpolarizations than those innervating fast muscles; this longer period, during which the membrane potential is more negative than the resting potential, contributes to a longer refractory period during which action potentials cannot be stimulated. A single motor neuron innervates several muscle fibers, and this combination of a motor neuron and its fibers is known as a motor unit (MacIntosh et al., 1996); Burke et al. (1971) synthesized the physiologic and histochemical characteristics of motor units to determine that there are three non-overlapping types of motor units in the cat gastrocnemius. One type of motor unit labeled "S", for "slowly contracting", had a high fatigue resistance (held >75% of initial tension output after 2 minutes of repetitive stimulus), a relatively large twitch contraction time

(corresponds with low velocity), relatively low maximum tetanic tension, and a distinct pattern of ATPase staining; another type labeled "FF", for "fast contracting, fast fatigue", had low fatigue resistance (held <25% of initial tension output after 2 minutes of repetitive stimulus), low twitch contraction time (corresponds with high velocity), relatively high maximum tetanic tension, and distinct patterns of ATPase staining; lastly, an intermediate type labeled "FR", for "fast contracting, fatigue resistant", exhibited relatively high fatigue resistance (held >75% of initial tension output after 2 minutes of repetitive stimulus), intermediate contraction time, relatively low tetanic tension, and a distinct ATPase staining pattern.

MacIntosh et al., 1996, synthesized information from experiments and classification schemes by Burke et al. (1971), Peter et al. (1972), and Brooke and Kaiser (1970), and described three different types of motor units and their properties, and is reproduced in Table 1.1. It is important to note that not all fibers fit neatly within these categories, and, further, a single fiber may contain many different isoforms of myosin heavy chain (MHC), which contains the ATPase enzyme, in both muscles transitioning between type and in those in a steady state; previous experiments may have misinterpreted fibers with mixed MHCs to be distinct fiber types, (*as reviewed in* Pette et al., 1991). In their review of single fiber analysis, Pette et al. (1991), however, suggest that a standardized classification system based on MHC is probably warranted, as a correlation of MHC with mATPase activity, contractile speed, the amount of ATP and PCr in a fiber, the ability to phosphorylate ATP (as implied by ATP/ADP), and with tension costs (amount of ATP per unit of isometric force) suggests that MHCs may be a more fundamental basis of organization.

Table 2.1 - The three types of motor units and their properties, as synthesized by MacIntosh et al. (1996).

Property	Motor Unit Type I	Motor Unit Type IIA	Motor Unit Type IIB
Twitch speed	Slow	Fast	Fast
Twitch force	Small	Intermediate	Large
Fatigability	Low	Low	High
Red Color	Dark	Dark	Pale
Myoglobin	High	High	Low
Capillary Supply	Rich	Rich	Poor
Mitochondria	Many	Many	Few
Z-line	Intermediate	Wide	Narrow
Glycogen	Low	High	High
Alkaline ATPase	Low	High	High
Acid ATPase	High	Low	Moderate
Oxidative enzymes	High	Medium-High	Low

Histochemical tests for myosin ATPase, identification of MHC by gel electrophoresis, western blot or liquid chromatography-tandem mass spectrometry, and determination of metabolic phenotype via enzyme assays are the most commonly used methods for describing fiber type (Scott et al., 2001). The histochemical test for ATPase is based on the fact that the ATPase of fast-twitch muscles is denatured in acidic conditions, while the ATPase of slow-twitch muscles is denatured in basic conditions. Thus, if samples are pre-incubated in basic pHs, only fast-twitch muscles will display ATPase activity, which produces ADP and P; samples are probed for P in this procedure, which stains darkly in fibers with the fast-twitch myosin ATPase phenotype. Slow-twitch muscles will stain dark if the sample is pre-incubated in an acidic pH. Identification of MHC isoforms is another method used for fiber-typing. If myosin controls are available, isoforms of myosin in a sample can be distinguished via gel electrophoresis (Roberts et al., 2012). If a positive control is not available, with the appropriate antibodies, immunocytochemical staining or western blots can be used to identify myosin isoforms (Feldman and Stockdale, 1990; Dusterhoff and Pette, 1992). Lastly, samples can be tested via liquid chromatography – tandem mass spectrometry for the presence of myosin isoforms (Shero et al. 2012). Metabolic enzyme concentrations, in tandem with ATPase activity, are also used to

classify fiber types; Burke et al. (1971) used this approach when describing three distinct types of motor units.

Known molecular regulators of fiber type determination

Chin et al. (1998) hypothesized a regulatory role for calcineurin, a serine/threonine phosphatase, in fiber-type expression, based on the observations that a) slow-twitch muscles are innervated by neurons that fire consistently and slowly, producing sustained, low amplitude intracellular calcium levels, as compared to the fast twitch muscles, that produce fleeting, high amplitude calcium levels, and b) in lymphocytes, calcineurin responds to only the sustained, low amplitude pattern of calcium signaling, and activates the nuclear factor of activated t-cells (NFAT) and myocyte enhancer factor-2 (MEF2). They found that, in c2c12 cells, constitutively active calcineuirn increased the transcription of myoglobin and troponin 1 slow (TnIs) promoters, which they consider specific to MHC I, and did not increase transcription of fast-fiber type specific genes; cells with inhibited calcineurin did not transcribe the myoglobin gene, and a calcineuirn inhibitor prompted an *in vivo* slow to fast fiber type change in mice. In addition, the authors found that the interaction of multiple transcription factors, not just NFAT, is necessary for the expression of myoglobin. Naya et al. (2000) produced similar results, and noted that, in addition to increased transcription of myoglobin and TnIs, expression of slow MHC (as measured by ATPase activity) and the transcription of sarcomeric mitochondrial creatine kinase increased with calcinuerin activity. Lin et al. (2002) found that the peroxisome-proliferator-activated receptor-g coactivator-1 (PGC-1α), known to stimulate mitochondrial biogenesis, was also involved in fiber-type determination. PGC-1α appears to co-stimulate transcription factors

in the calcineurin pathway, specifically, MEF2 isoforms, and is itself influenced by calcineurin, and other, unknown pathways.

Using c2c12 cells, Allen et al. (2001) determined that the promoters of the three isoforms of fast twitch muscle (IIa, IIx, and IIb) shared four similar motifs, a TATA box, a CArG box, and two AT-rich motifs, and all isoforms had several potential NFAT and MRF binding domains. Although all isoforms were found to have MRF binding domains, MRF only increased the promotor activity of MHC IIb. Calcineurin increased the promoter activity of IIa significantly more than it did IIb, but no more so than IIx. NFAT increased IIa promotor activity and decreased IIb and IIx promoter activity, however, mutation or deletion of NFAT and MEF2 binding motifs on the promoters still resulted in IIa promoter activity when exposed to overexpressed calcineurin, so there are other factors involved. Lastly, the effect of MyoD and MEF2 was tested in non-muscle isoforms, to try to isolate its effect from any inhibitory effects of the c2c12 myotube milieu, *i.e.* overexpression and "saturation" of said factors; MEF resulted in an increase in all three promoters, while MyoD increased IIb activity more than IIx or IIa.

Influences of the muscle of origin on fiber type in primary myotubes

While the influence of pathways dependent on neuromuscular (calcium) signaling have been elucidated, studies examining the influence of the fiber type of the donor muscle on the fiber type of myotubes derived from their satellite cells have produced conflicting results. Roseblatt et al. (1996) found that satellite cell clones derived from the primary culture of adult mouse muscle tissue exhibited distinct fiber types, as defined by myosin heavy chains,

depending on the fiber type of the muscle of origin after seven days of differentiation. This is not to say the fiber types of the satellite cells were the same as those of the muscle of origin - they did not find any MHC IIa or IIb fibers in satellite cells derived from either slow or fast twitch fibers; instead, they found that myotubes derived from slow muscles (*soleus m*) produced type I MHC, while those derived from fast muscles (*extensor digitorum longus m*) did not express type I MHC. In this study, the muscle fiber from which the satellite cells originated were kept in culture with the cells for four days during proliferation; perhaps the fibers themselves contained factors that help determine final fiber type. Barjot et al. (1995) examined rabbit skeletal muscle primary culture, and also found differences in MHC type expressed in myotubes based on the muscle of origin's fiber type, and found that the timing of expression of growth factors and proteins, including the MHCs, varied depending on fiber type. Dusterhoff and Pette (1990) found that satellite cells grown from soleus and tibialis muscle of rats reflected their parent muscle's fiber type, but only if grown on matrigel. Further, both Dusterhoff and Pette (1990) and Ghosh and Dhoot (1998) noted that clones passaged several times could change their fiber type. Hughes and Blau (1992) found that, while myoblasts from a satellite cell clone of an adult rat formed myotubes that did not express an MHC corresponding to Type IIA fibers, the clones were able to fuse with *in vivo* fibers that both did and did not express this MHC, suggesting any intrinsic inclination for fiber type can be influenced by the cells' environments. Finally, LaFramboise et al. (2003) found that satellite cells derived from the primary culture of adult rat tissue did not reflect the fiber type and phenotype of the muscle from which they were derived; all cultured cells co-expressed mostly MHC IIa and MHC Ia on day three and day six of differentiation. In all of the aforementioned primary culture studies, growth and differentiation media were based on either Dulbecco's Modified Eagles Medium, Minimum Essential Media, or

HAM's F10 media, and supplements varied; most notably, LaFramboise et al. (2003) were the only ones to report using insulin in their differentiation media, and they were the only ones that strictly observed no difference in fiber type between myotubes originating from different fiber types . Taken together, these studies strongly suggest that cell culture environment must be considered when studying fiber type *in vitro.*

Influences on fiber type in c2c12 cultures

The fiber type of c2c12 cells has been described as 100% type IIa (Laframboise et. al. 2003) or mixed type I and II (Yuan et al., 2011; Zebedin et al. 2004). LaFramboise et al (2003) found that, on day three and day six of differentiation, c2c12 cells expressed mostly type IIa myosin heavy chain (MHC). Yuan et al. (2011), in their study examining the relationship of the forkhead box 01 promoter with fiber type, reported that on day 3 of differentiation, the fiber type of control c2c12 cells was mostly fast, and, furthermore, RNA data showed mostly type IIx, although some type IIb and type I were also present. Zebedin et al. (2004), who were investigating the effects of fiber type conversion on currents in voltage-gated sodium channels, found that after 17 days of differentiation, myotubes of their c2c12 control group expressed both fast and slow fiber types; immunoblot data suggest an almost equal amount of slow and fast MHCs. These experiments had variations in plating substrate, growth media, and experimental design that cannot be excluded as possible influences on fiber type. Dusterholf and Pette (1993) reported that myotubes derived from rat soleus muscle developed only embryonic myosin when grown on gelatin, but expressed adult type I myosin when grown on Matrigel (Laminin, Entactin, Collagen IV, and several growth factors). Ghosh and Dhoot (1998), however, reported that the

substrate did not affect the proportion of slow MHCs in myotubes derived from fetal human quadriceps muscles, although matrigel did improve myoblast proliferation as compared to gelatin. Further, slow MHCs have been reported in cultured myotubes derived from several species and grown on gelatin, collagen and matrigel, suggesting that plating substrate, while possibly influential, might not be a major determinant of fiber type in a cultured myotube (Feldman and Stockdale, 1990; Barjot et al. 1995; Laframboise et al. 2012). As with the primary culture studies, supplemental factors in the differentiation media varied, most notably insulin, which was only used in the study by Laframboise et al. (2003), and these cells did not produce any slow fibers.

Materials and Methods

Weddell seal muscle biopsy collection methods, as well as primary myoblast isolation and culture methods have been described previously by Garcia et al. (in review). Four treatment groups each for c2c12 and seal cells were established; for each species, cells were grown and differentiated in media with either 2.5% or 5% lipid, and were differentiated in either normoxic (21% O_2 ; 5% CO_2) or hypoxic (0.5% O_2 ; 5% CO_2) conditions. As Weddell seal cells do not proliferate without lipid beyond initial plating (Garcia et al., in review), cells were not grown in this condition to conserve material. Within a treatment group, both growth and differentiation media were supplemented with the same amount of lipid.

C2C12 and Primary Muscle Cell Culture

Cells were proliferated in their respective oxygen and lipid treatment groups for 8 days. Cells were proliferated in high-glucose Dubelcco's modified growth media (DMEM; Sigma Aldrich, product # D5671, St. Louis, MO)- based growth media, supplemented with 20% FBS (Atlanta Biologicals, product # s11050, Flowery Branch, GA) 1% antibiotic/antimycotic (Life Technologies, product # 15240-062, Grand Island, NY) 1% sodium pyruvate (Life Technologies, product # 11360-070, Grand Island, NY), and either 2.5% or 5% lipid (Life Technologies, product # 11905-031, Grand Island, NY) depending on the treatment group. All treatment groups were proliferated in an incubator at at 37° C, 21% O_2, and 5% CO_2. Cells were split when plates were 40%-60% confluent, and trypsin was not used. Instead, cells were washed twice in PBS (Sigma Aldrich, product # P3813, St. Louis, MO) incubated in PBS for 3 minutes, and then gently scraped from the bottom of the plate.

Differentiation was initiated at approximately 90% confluence by switching to a high-glucose DMEM based media supplemented with 2% Equine serum (Life Technologies, product # 16050-122, Grand Island, NY), 1% antibiotic/antimycotic, 10 μg/ml insulin (Life Technologies, product # 12585-014, Grand Island, NY), 10 μg/ml transferrin (Rockland, product # 009-0134, Gilbertsville, PA) and the appropriate amount of lipid for the treatment group (2.5% or 5%). At this time, plates in the hypoxic treatment group were moved to a hypoxic chamber (0.5% O_2, 5% CO_2, 37° C); plates in the normoxic treatment group were kept in the incubator with aforementioned settings.

Cells were differentiated for 7 days (approximately 168 hours), and then 1-2 plates from each treatment group were harvested with one of two homogenization buffers. Plates with myotubes to be used for myoglobin assays or western blot were harvested with a Tween (Fisher-Scientific, product # BP377-500, Pittsburgh, PA) based lysis buffer composed of 79% PBS, 20% Glycerol (Macron, product # 5092-02, Omaha, NE) and 1% Tween. Plates with myotubes to be used for gel electrophoresis and Coomassie staining were harvested with 500 µl homogenization buffer based on a formulation by Roberts et al. (2012), and consisting of Tris-HCl (Corning, product # 46-030-CM, Corning, NY), pH 6.8, 5% 2-mercaptoethanol (Acros Organics, product # 12547, New Jersey) 2.5% glycerol, and 2.3% sodium dodecyl sulfate (Acros Organics, product # 22614-5000, New Jersey). After harvesting, protein homogenate was heated at 60° C for 10 minutes, and frozen at -80° C.

Protein assays

Cells harvested with the Tween-based buffer were assayed via a Bradford assay kit (ThermoScientific, product # 23236, Rockford, IL) to determine protein concentration, with some modifications to reduce sample volume used. After establishing a standard curve, 20 µl sample were added to 980 µl Coomassie Brilliant Blue incubated for 10 minutes, and read at 595 nanometers (Beckman-Coulter, DU-800 Spectrophotometer, Brea, CA).

Cells harvested with buffer based on the Roberts et al. (2012) formulation were assayed via the RC/DC protein assay protocol provided by BioRad (product # 500-0122, Herculus, CA), with the following modifications; 1) Sample and reagent amounts were modified proportionally

to fit a 1 ml cuvette; 2) samples were centrifuged for 10 minutes instead of 2-5 minutes. Samples were read at 750 nm (Beckman-Coulter, DU-800 Spectrophotometer, Brea, CA).

Gel electrophoresis

The protocol of Roberts et al. (2012) was used to hand-cast gels, with some modifications. The separating gel was made by adding 2.25 ml resolving buffer (16.2 g Tris (Avantor, product # 4109-02, Center Valley, PA),5.0 g glycine (EMD Millipore, product # 570-500GM, Billerica, MA), mixed with 100 ml diH_2O; 2.7 g of sodium dodecyl sodium (SDS; Acros Organics, product # 230425000, New Jersey) was then added, and the pH adjusted to 8.80), to 8.75 ml 51.4% glycerol (brought up in diH_20), and 4ml 30% acrylamide (29.4 g acrylamide (Sigma Aldrich, product # A8887, St. Louis, MO) ; 0.6 g bisacrylamide (BioRad, product # 16102011, Hercules, CA); 29:1 acrylamide:bisacrylamide), and brought up to 100 ml in diH_20). After mixing the separating gel together, 150 µl 10% ammonium persulfate (BioRad, product # 161-0700, Hercules, CA; brought up in diH20) and 15 µl tetramethylethylenediamine (TEMED; EMD Millipore, product # 8920-OP, Billerica, MA) were added, and the entire mixture was gently swirled for 20-30 seconds. The mixture was added to the plates, and topped with 2 ml diH_20 to level the gel, and left to set for 1 hour.

After the separating gel set, the diH_20 was removed and the separating gel was added, along with a 10 or 15 well comb. The stacking gel was made by mixing 1.02 ml stacking buffer (3.0 g Tris, with 0.4 g ethylenediamminetetraa-cetate (EDTA; Sigma Aldrich, product # ED-100G, St. Louis, MO), brought up to 100 ml in diH_20, after which 2.5 g of SDS was added, and

the mixture adjusted to pH of 6.8), 2.19 ml 51.4% glycerol, and 0.5 ml 30% acrylamide. After the mixing the stacking gel together, 75 µl 10% ammonium persulfate, and 7.5 µl TEMED were added, and the entire mixture was swirled for 20-30 seconds. The gel was set for 1 hour.

After the gel was set, lower running buffer (100 mM Tris, 150 mM glycine, and 0.1% SDS) was added to just below the wells, and enough upper running buffer (500 mM Tris, 750 mM glycine, 0.5% SDS, % 2-mercaptoethanol) was added such that the wells were full. Samples were mixed 1:1 with Lamelli buffer (62.5 mM Tris-HCL, 25% glycerol, 2% SDS, 0.01% Bromophenol (BioRad, product # 161-040, Hercules, CA), in diH_20, pH 6.8) supplemented with 5% 2-mercaptoethanol, heated for 2 minutes at 100° C, and placed on ice. 15 µg sample were loaded per well, as was 10 µl of pre-stained protein ladder (Fisher Scientific, product # SM1811, Pittsburg,PA). Samples were separated at 190 V for approximately 5 hours. Care was taken such that the ladder rung associated with 250 kD traveled the same distance on each gel; thus the separation time varied slightly.

After electrophoresis, the gels were removed and washed in DiH_2O for 45 minutes. Gels were then stained in Coomassie Brilliant Blue overnight (approximately hours). Gels were destained (75 ml 1.0 M acetic acid (VWR, product # VW0125-3, Radnor, PA) , 400 ml methanol (Fisher Scientific, product # A4574, Pittsburgh, PA), 525 ml diH_2O) 2 times, once for 5 minutes, and then again for 3 hours. After destaining, gels were imaged with a BioRad ChemiDoc XRS+ imaging system (Hercules, CA).

Western Blots

Protein homogenized with the Tween-based lysis buffer was used for immunoblotting. SDS-PAGE (200 µl 20 mM Tris (pH 8.0), 6 ml 6% SDS, 2 ml glycerol, 1 ml 10% 2-mercaptoethanol, 200 µl 10 mM EDTA, 600 µl diH_2O, 0.1 mg 0.01% bromophenol blue) was added to samples in a 1:1 ratio, and the mixture was boiled for 3 minutes. Afterward, it was immediately placed on ice for 2 minutes, and then centrifuged in a glass-wool column for 30s at 16,100 rcf. Samples and a pre-stained ladder were run at 150v for 45 minutes. Samples were transferred to nitrocellulose paper with via dry transfer (iblot, Invitrogen) over 7 minutes. Samples were rinsed in TBS/T (2.42 g Tris Base, 8.0g NaCL (VWR, product # 6530-5, Radnor, PA), brought to 1 liter in diH_2O pH 7.6, with 1 ml Tween added), for 5 minutes, and then incubated in 5% milk in TBS/T for 1 hour. After another TBS/T rinse, samples were incubated with antibodies raised against either MHC I, MHCIIa, or MHC IIb at 1:10 dilution with TBS (Developmental Studies Hybridoma Bank, product names S22 (42 µg/ml), SC-71 (55µg/ml), BF-F3 (63 µg/ml), respectively, Iowa City, IA). Samples were then rinsed 3x for 4 minutes with TBS/T, and then incubated in a 1:3000 dilution of secondary antibody (1:5000 dilution; Pierce, product # 31432, Rockford,IL) with TBS/T brought up in 5% milk. Samples were again rinsed 3x for 15 minutes in TBS/T before being developed for 3 minutes (SuperSignal West Dura Chemiluminecent Substrate, ThermoScientific, product # 34075, Rockford,IL) and imaged.

Liquid Chromatography Tandem Mass Spectrometry

One band representing each of the purported MHC isoforms was excised for each species (3 total for c2c12, 2 total for seal). Samples were sent to the Proteomics and Metabolomics Facility at Colorado State University, where bands were digested with trypsin, and separated via liquid chromatography (EASY nano-LC, 3µm, 75 µm ID x 100mm C18 column; Thermo Scientific, Rockford, IL) for 25 minutes at a flow rate of 400 nl/min via a linear gradient from 10% to 30% B buffer (100% Acetonitrile,0.1% formic acid). Samples were input directly after chromatography into the mass spectrometer (Orbitrap Velos; Thermo Scientific, Rockford, IL), where spectra were collected over a m/z range of .4-2 kD using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Xcalibur 2.2 software (Thermo Scientific, Rockford, IL) with a S/N threshold of 1.5 and 1 scan/group was used to produce compound lists of the resulting spectra.

Mass spectrometry data were loaded, along with the Uniprot Murine database, into Scaffold Proteomics Software V.4.0 9 (Proteome Software; Portland, Oregon). Only proteins whose peptides passed a 95% threshold as calculated by the Scaffold Local FDR algorithm, had at least 2 identified peptides, and a protein identification probability of greater than 95% were considered. Percent coverage, which is the percent of amino acids that make up the protein that were detected in the sample, and the exclusive unique peptide count were assessed to determine data quality for MHC isoforms.

The normalized spectral abundance factor (NSAF) was calculated for each protein to determine band composition. It is important to note that the NSAF values calculated by Scaffold Proteomics Software are **not** NSAF values as originally defined by Zybailov et al. (2005) (Susan Ludwigsen, bioinformatics analyst, Proteome Software; personal communication). NSAF was defined by Zybailov et al. (2005), as follows:

$$NSAF_k = \frac{(\frac{Spc}{L})_k}{\sum_i^N (\frac{Spc}{L})\, i}$$

Here, the spectral counts associated with a protein k (Spc) are divided by the length, defined as the number of amino acids found in that protein (L); this is the spectral abundance factor (SAF). Then, the SAF for all N proteins in each sample are summed, and the SAF for a protein k is divided by this sum; this is the NSAF for protein k.

In contrast, the Scaffold algorithm calculates SAF values for each protein, and then normalizes *across samples*, instead of within a sample, such that the SAF value is the same for all samples loaded in an experiment window. In other words, if data from all 5 excised bands in this experiment were loaded into an experiment window, it would calculate a factor that would ensure that all 5 bands had the same summed SAF; this would be the normalization step. If only one sample is loaded into an experiment window, then only SAF is calculated. For this experiment, we used the NSAF as defined by Zybailov et al. (2005), thus, only one sample was loaded into an experiment window at a time, and the SAF was calculated by Scaffold. These data were then exported to excel, where SAF was summed for the one sample and used to find NSAF for all suitable proteins in that sample.

Myoglobin

A method modified from that of Reynafarje (1963) was used to determine myoglobin concentration in each plate. Samples were added to 0.04 M potassium phosphate (kphos) buffer in a 1.8:1 ratio. This mixture was centrifuged for 50 minutes at 28,000xg. The supernatant was decanted, and each sample was bubbled for 3 minutes with carbon monoxide gas. A parallel experiment was conducted in which 0.01 g of sodium dithionite were added to each sample after 3 minutes of bubbling to ensure complete reduction; these samples were bubbled for an additional 2 minutes after the addition of the dithionite. Samples were read in triplicate by a Biotek Synergy HT plate reader (Winooski, VT) at 538 and 568 nanometers. The following formula was used to determine myoglobin concentration:

$$\text{Myoglobin}_{(mg/ml)} = (538_{nm}-568_{nm})*5.685*V1/V2*\text{sample dilution factor}$$

Here, V1 = the volume of sample plus the volume of kphos added to it, and V2 = volume of sample added to the kphos. This value was normalized by protein concentration. ANOVA was used to check for significance between treatment groups, and was considered at $p<0.05$. To test for a significant difference between myoglobin values with and without dithionite, an unpaired Student's t-test was performed.

Results

Gel electrophoresis

Gels for each treatment group are shown in Figure 2.1 The c2c12 hypoxia, A24 normoxia, and A24 hypoxia treatment groups, for all lipid concentrations, resolved 2 distinct bands at approximately 220 kD. C2c12 normoxia resolved 3 distinct bands for both treatment groups, but this result was not reproducible; subsequent gel separations for this treatment group only resolved 2 distinct bands. While care was taken such that the gel fronts ran the same distance in all treatment groups for a given experiment, it is important to note that gel fronts were allowed to run further down the gel in later experiments than in initial ones, and Figure 2.1 depicts some bands from initial and later experiments. Regardless, all runs resolved at least 2 bands for each treatment group.

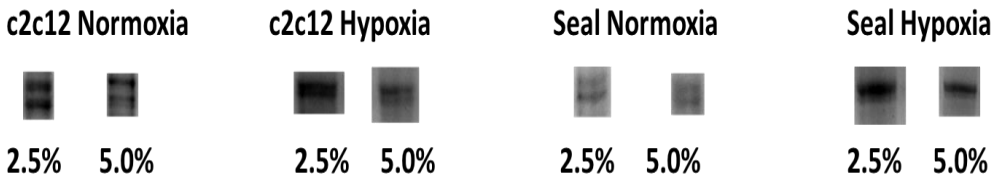

Figure 2.1 – Gel electrophoresis and Coomassie staining resolved two bands in all treatment groups, with the exception of one run of c2c12 normoxia 5.0%, which yielded three bands.

Protein identification via Western Blot

None of the adult MHC isoforms probed for were consistently detected in either c2c12 or A24 samples. A probe for MHC IIa in c2c12 reacted during two runs, but this was not reproducible in later experiments.

Protein identification via LC-MS/MS

All detected MHC isoforms had greater than 10% representation, in terms of amnio-acid percent coverage. With the exception of MHC IIb and MHC slow/cardiac beta, MHC isoforms identified in each sample had ≥ 5 exclusive unique peptides associated with them (Figure 2.2).

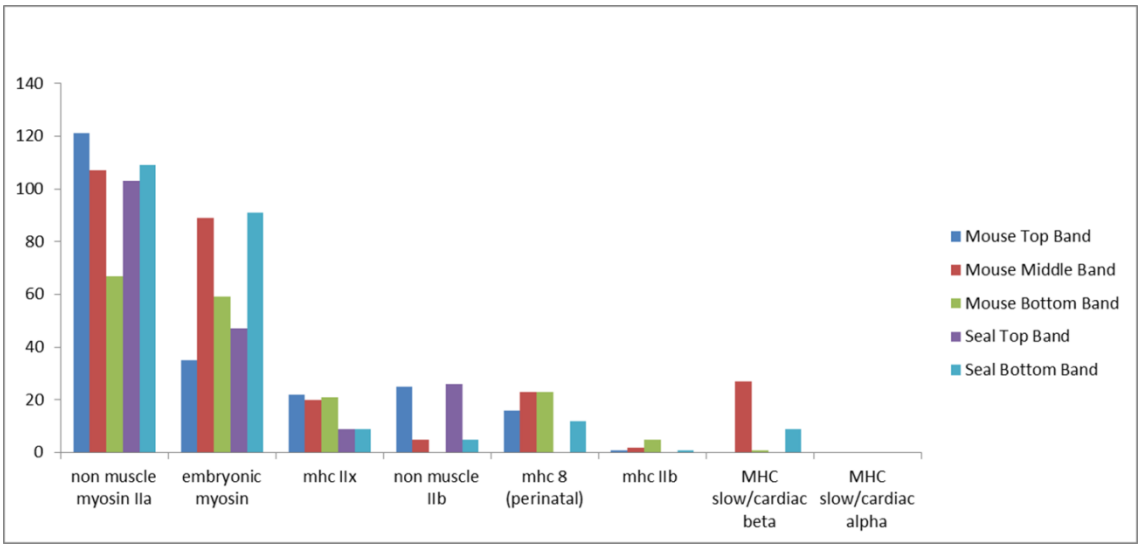

Figure 2.2 – The exclusive unique peptide count was used as an indicator of data quality, and was ≥ 5 for all myosin isoforms except MHC IIb and MHC slow/cardiac beta.

All bands sent for proteomic analysis were heterogeneous in terms of protein composition, as discerned by NSAF, and there was very little detectible adult MHC (Figure 2.3).

The top-most c2c12 band (corresponding with relatively heavier proteins) was composed of 46% MHC

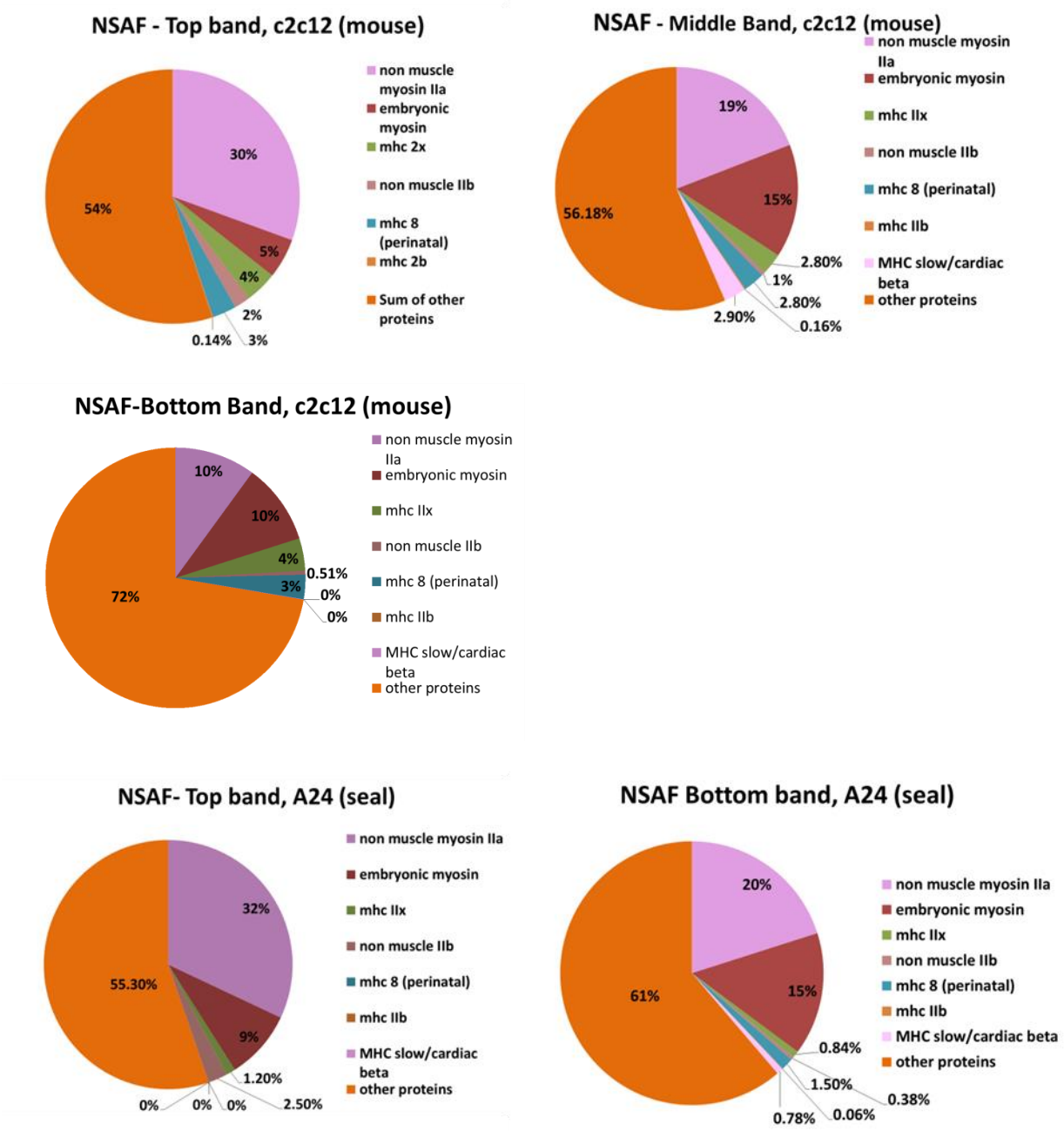

Figure 2.3 – NSAF breakdown for observed bands in each species. Protein composition was heterogeneous, and adult MHC isoform presence was minimal.

isoforms, but, most of that was non-muscle myosin IIa (30% of total); the most abundant adult MHC was MHC IIx, at 4%. The middle c2c12 band was composed of 44% MHC isoforms, but, the most abundant MHC isoforms were non-muscle myosin IIa (19% of total), and embryonic myosin (19% of total); the most abundant adult MHC was slow/cardiac alpha (2.9% of total), closely matched by MHC IIx (2.8% of total). The bottom c2c12 band was composed of 28% MHC isoforms, and, again, most of that was composed of non-muscle myosin (10%) and embryonic myosin (10%). The top seal band was composed of 45% MHC isoforms, and, as is in the c2c12 samples, most of that was composed of non-muscle myosin IIa (32% of total); the most abundant adult MHC isoform was MHC IIx, at 1.2% of total. The bottom seal band was also composed of about 39% MHC isoforms, with non-muscle myosin IIa (20% of total) and embryonic (15% of total) being the most abundant.

Myoglobin

Myoglobin was detected in all treatment groups (Figure 2.4). There was no significant

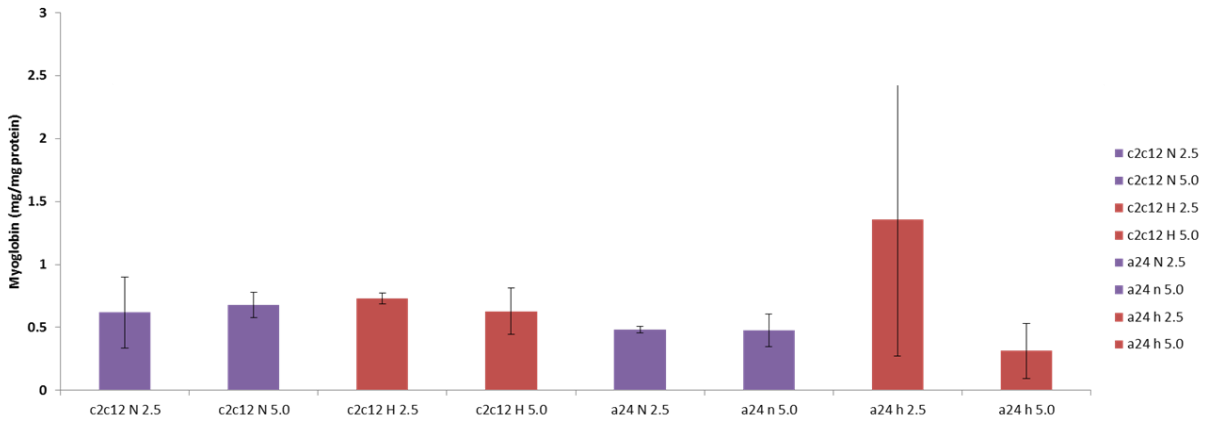

Figure 2.4 – Average myoglobin in each treatment group. There was no significant difference between any treatment groups ($p > 0.05$, error bars are S.E.M., n=3 for all except c2c12 H 2.5).

difference between any treatment groups. The addition of dithionite, to ensure a complete reduction, slightly increased myoglobin values compared to samples without dithionite, but this was not significant. There was no significant difference between treatment groups with the addition of dithionite.

Discussion

Proteomic analysis revealed gel bands with heterogeneous protein compositions, and minimal adult MHC in both the seal and c2c12 samples. These results partially support those of Rosenblatt et al. (1996), who, on day 7 of differentiation, found that the primary cell culture of both fast and slow adult skeletal muscle produced myotubes lacking in adult MHC IIa and IIb isoforms, but rich in embryonic and neonatal MHC. However, while Rosenblatt et al. (1996) detected slow MHC in the majority of myotubes derived from slow muscle, we found less than 1% in our seal sample bottom band, as assessed by NSAF values. The fiber type composition of the *longissiumus dorsi m* of the adult male from which this sample was taken was not assessed, but previous studies of Weddell seal *longissiumus dorsi m* have found the muscle to be composed of approximately 80% MHC I and approximately 20% MHC IIa, with a near absence of MHC IIb; thus, if there is parent-muscle dependent fiber type expression in cultured cells, as described by Rosenblatt (1996), this is not an influence here (Kanatous et al., 2008). Additionally, our study found type II adult MHC, however minimally, in contrast to the findings of Rosenblatt et al. (1996). Our results also partially support those of Barjot et al. (1995), who assessed the fiber type composition of cultured primary myotubes derived from rabbit muscles. Barjot et al. (1995) cultured cells from the slow-twitch *semimembranosus proprius m* and fast-

twitch *semimembranosus accessorius m* of 3 month old rabbits, and detected adult fast and slow MHC, as well as neonatal MHC, on days 5, 8, and 11 of differentiation for the slow twitch muscle, and neonatal and fast MHC on days 8 and 11 for the fast twitch muscle. While we detected all three of these isoforms in our samples, harvested on day 7 of differentiation, we found more embryonic than adult MHC; looking at the qualitative Western data presented by Barjot et al. (1995), it is hard to discern if this pattern holds for their samples.

On day 6 of differentiation, LaFramboise et al. (2002) harvested several muscles of fast, slow, and mixed fiber type from adult mice, and found that primary myotubes derived from these muscles, regardless of parent fiber-type, expressed mostly MHC IIa, followed by MHC I, with little to no amount of IIx, IIb, and neonatal MHC; this conflicts with our findings in that we found no MHC IIa. Interestingly, the antibody they used to probe for MHC IIa, SC-71, was the same one that was used in our study, and we found a weak reaction with c2c12, although this result was not reproducible in subsequent tests in our lab. While our c2c12 cells had small amounts of adult MHC IIx, and trace MHC IIb, and similarity in the amino acid composition of all muscle MHC isoforms is expected, given the specificity of the SC-71 antibody (Schiaffino et al., 1989), the likelihood of cross-reactivity with other adult MHC is low, and non-specific staining seems to be the most reasonable explanation.

Our results also conflicted with the conclusions of LaFramboise et al. (2002) regarding c2c12, who found that c2c12 myotubes expressed exclusively MHC IIa on day 2 and day 6 of differentiation. This was confirmed via co-migration of a c2c12 band with that of MHC IIa from another immortalized mouse myoblast culture (MM14), which was itself initially assessed via

immunoblot. This was not consistent with the results of the present study; using proteomics, we found no MHC IIa in c2c12 cells, and, as with the seal cells, very little adult MHC. In contrast to LaFramboise et al. (2002), Yuan et al. (2011) found both fast and slow MHC in control c2c12 cells differentiated for only 3 days, however, the anti-fast antibody they used, MY-32, according to the manufacturer's product description and published studies, stains both fast and neonatal isoforms (Product # M4276, Sigma Aldrich, St. Louis, MO; Harris et al., 1989). Thus, it is possible that they may have detected slow and neonatal isoforms on day 3, however, their RNA analysis, which will be discussed in subsequent sections, did detect fast MHC transcription, so it is unlikely that they only detected neonatal isoforms. Zebedin et al. (2004), who also found fast and slow MHC in their unstimulated control c2c12 cells after 6 and 17 days of differentiation, also used the MY-32 antibody to detect fast MHC isoforms. If these two studies inadvertently detected neonatal/perinatal MHC isoforms, instead of fast adult MHC, along with the adult slow isoform, this would be more constant with the findings of our study, which found small amounts of adult slow/cardiac beta MHC (3% in the middle band, as determined by NSAF), along with small amounts of perinatal MHC (3% in all three bands, as determined by NSAF). Lastly, via western blot, Allen et al. (2001) studied fast isoform promotors and protein expression in c2c12 cells, and found that the IIx isoform was most expressed, followed by IIb, and IIa, thus supporting our results; promotor activity matched this trend.

Although our study did not detect much adult MHC protein on day 7, it is possible that relatively significant amounts of mRNA for these isoforms is transcribed by this stage in development, but is not yet translated. While our study did not include RNA analysis, and, to the author's knowledge, there has not been a high resolution time-series analysis of RNA expression

in primary skeletal muscle or c2c12 cells during differentiation, two of the aforementioned studies examined RNA for c2c12 cells at two time points, on day 3 (Yuan et al. 2011), and day 6 (LaFramboise et al. 2003). In their control c2c12 cells, Yuan et al. (2011) found that MHC IIx had the highest relative mRNA levels, followed by MHC IIb, at about ¼ of MHC IIx, and then by MHC I, which had ~ 1/8 of MHC IIx levels. They detected no MHC IIa RNA. Looking at MHC protein expression via western blot, however, suggests that there are similar amounts of MHC I and MHC IIx being expressed. While speculative due to the qualitative nature of the western blot, this may suggest that some IIx mRNA is either not being translated, or subject to substation post-translation modification. LaFramboise et al. (2003) found MHC IIa to be most abundant in c2c12 on day 6, with >75,000 phosphor intensity, vs. <25,000 for all other isoforms. In terms of protein expression, they detected only MHC IIa; while mRNA expression of other isoforms was minimal, this study is another example of mRNA not completely reflecting protein detected via western blot. Both of these studies suggest that it is possible that adult MHC isoforms are being transcribed to mRNA, but not necessarily translated into protein as detected by antibodies. A time-series of RNA and protein expression is needed to further elucidate patterns of MHC isoform development in cultured myotubes.

The author is not aware of any proteomics-based studies focused solely on MHC isoform expression in cell culture. Kislinger et al. (2005), used a shotgun proteomics approach to assess protein dynamics during days 2, 4, 6, and 10 of c2c12 myotube differentiation. Using a database encompassing both the human and mouse genome (Swiss-Prot and TrEMBL), supplemental data provided by the authors shows that the skeletal muscle MHC isoform that was most abundant (in terms of spectral count) was identified as the human embryonic isoform (MYH3_Human).

Interestingly, it had spectral counts of 9, 17, 21, and 5 on days 2, 4, 6, and 10, respectively, indicating an increase at a similar time in which the myotubes of the present study were harvested, followed by a decrease sometime after day 6. The next most abundant skeletal MHC was mouse cardiac muscle alpha MHC isoform, with counts of 0, 4, 4, and 5 on days 2,4,6, and 10, followed by human MHC I, with one count on day 4, and human fetal and perinatal MHC, with 1 count each on day 10. Because Kislinger et al. (2005) were focused on general protein dynamics, and samples were not enriched for myosin via methods such as gel electrophoresis, it must be noted that it is possible that spectral counts would have been higher for these bands were the study aimed specifically at MHC isoforms.

Perhaps the most interesting outcome of this study was the fact that myoglobin was detected, albeit in small amounts, in every treatment group, despite little to no presence of adult MHC isoforms. While myoglobin transcriptional activity and protein expression have been detected in other *in vitro* studies, thus far, there have not been any experiments that have compared myoglobin protein expression to MHC isoform expression in cell culture (Chin et al., 1998; Lin et al, 2002; DeMiranda et al., 2012; Schlater et al., in review). However, in their *in vivo* studies of the developing mouse hindlimb, Garry et al. (1996) found that adult MHC expression occurs before that of myoglobin; there is no myoglobin present in fetal stages, and it is not detected until 2 days after birth, while MHC I is detected, albeit minimally (+ out of ++++), on day 13.5 of the embryonic period, and increases after birth, and MHC IIa is detected first at birth (++ out of ++++), after which it too increases (Garry et al., 1996). Furthermore, in the lateral gastrocnemius of a mouse, myoglobin is most abundant in fibers expressing MHC I and MHC IIa, followed by those fibers expressing IIx, and it was not found in fibers expressing

MHC IIb; their studies of a rabbit hindlimb had the same findings for all but MHC IIx (Garry et al., 1996). It should be noted here that these data show that, while myoglobin is most abundant in mitochondria-rich fiber-types, it is not exclusively found in MHC I, as is sometimes assumed, *e.g.*, Chin et al., 1998.

In general, myoglobin protein expression in cell culture has been much lower than that detected in whole tissue; for example, DeMiranda et al. (2012) reported values between .01 and .075 mg of myoglobin / mg protein in adult cultured Weddell seal myoblasts, as compared to 55 mg of myoglobin / mg of protein in the swimming muscles of adult Weddell seals (Kanatous et al., 2008; Schlater et al., in review). The myoglobin concentration in our seal cells was minimally higher when compared to whole tissue values, and ranged from 0.31-1.3 mg of myoglobin/ mg of protein. While there are not published data for mouse myoglobin values, the *vastus m* of a cotton rat has 1.7 mg of myoglobin/ mg of protein, while our values from c2c12 culture ranged from 0.62-0.72 mg of myoglobin/ mg of protein. Given these data, and developmental information of Garry et al. (1996), there are two possible explanations: 1) We only measured one time point, and thus, it is possible that, in our study, MHC did express first, and there is not much myoglobin because there is not much MHC; or 2) If robust MHC expression truly occurs before myoglobin expression, as in the manner of Garry et al. (1996), our results, in which myoglobin expression occurred before MHC expression, suggest the existence of a previously undescribed myoglobin regulatory mechanism.

Studies have confirmed the regulatory role of calcineurin in myoglobin expression and fiber-type determination, and have deemed the myoglobin promotors to be specific to MHC I

(although, again, it is found in MHC IIa and, to a lesser extent MHC IIx). However, Kanatous et al. (2008) found that myoglobin decreased in mouse tibilais anterior cells in hypoxic conditions (and no second stimulus) without a concurrent change in fiber type (Chin et al., 1998; Naya et al., 2000). Thus, while a calcineurin-based pathway links myoglobin and fiber-type, and a fiber-type dependent pathway undoubtedly affects, and is probably the most important factor in myoglobin expression, evidence implies that a fiber-type independent pathway may also exist, and that this might explain the presence of myoglobin, though minimal, in our study. The results of Schlater et al. (in review) suggests that this fiber-type independent pathway is stimulated by the addition of lipid to cell culture media, a factor that was present in all of our treatment groups. Schlater et al. (in review) compared myoglobin expression in c2c12 cells grown in 0% and 5% lipid; those grown in 5% lipid exhibited increased myoglobin expression, in both normoxic and hypoxic conditions. Schlater et al. (in review) hypothesized that the response might be attributed to a previously undescribed pathway in which lipid, either directly or indirectly, increases myoglobin concentration to facilitate ROS scavenging, a lesser-known property of myoglobin (*reviewed in* Garry and Mammen, 2007). When the authors added a ROS scavenger to the normoxic 5% lipid group, there was no longer a significant difference between it and the 0% treatment group, and adding a ROS scavenger to the 5% hypoxic treatment group decreased myoglobin significantly compared to the 5% group without the scavenger, but was still significantly greater than the 0% hypoxic treatment group. Moreover, Schlater et al. (in review) found that these increases in myoglobin did not correlate with calcineurin abundance, further suggesting a fiber-type independent pathway. While work remains to be done to unravel these pathways, their results support a new paradigm in which myoglobin expression is not solely dependent on fiber type.

Lastly, our myoglobin results did not replicate those of DeMiranda et al. (2012), in the sense that we had no significant difference between lipid and oxygen treatment groups, while they found a significant increase in hypoxic treatment groups (with a constant lipid concentration), and a significant increase in normoxic treatment groups with an increase in lipid concentration. Due to the concurrent culturing of our samples with those of primary cultures grown from biopsies attained from wildlife in a non-sterile environment, antimycotic was added to all treatments to prevent contamination; this was not done in the DeMiranda et al. (2012) study. The antimycotic, Amphotericin B, kills fungi primarily by binding to ergosterol, which is found in the lipid membrane of fungal cells; amphotericin B can also, to some extent, bind to cholesterol (Murray et al. 2005). Amphotericin B can also kill cells directly by forming free radicals, which can damage the cell membrane (Murray et al. 2005). One of the main ingredients in the supplemental lipid mixture is cholesterol, and, while it is not certain that this lipid contributes to the pathways suggested by Schlater et al. (in review), its removal from the system via binding by amphotericin B would deviate from the experimental conditions of both DeMiranda et al. (2012) and Schlater et al. (in review), who used the same lipid mixture. Given the low concentration of amphotericin B added to the cell culture (approximately 1.35* 10^-9 M) media relative to the amount of cholesterol in the supplemental lipid mixture (approximately 1.4*10^-5 M in the 5% treatment group), it is unlikely that the amphotericin B is binding up all the cholesterol, however, tests must be done to prove this doesn't have an effect. It is also uncertain if the amphotericin B is directly affecting membranes externally via ROS production.

Conclusions and Future Directions

Cultured c2c12 and seal skeletal muscle cells express myoglobin despite exhibiting minimal amounts of adult MHC isoforms. The amount of myoglobin expressed is less than that in whole tissue, and this may be due to either the dearth of adult MHC isoforms, or to the presence of a newly described, fiber type – independent pathway in which myoglobin expression is increased by the addition of lipid. These hypotheses should both be tested in future experiments, which should include a 0% lipid c2c12 cohort, compared against 2.5% and 5% lipid treatment groups. RNA and protein homogenate should be collected on every day of differentiation for each treatment group, so that mRNA, myoglobin protein, and MHC isoform expression can be tracked, and correlations of myoglobin transcription and expression to that of MHC and to lipid concentration clarified. If this step demonstrates myglobin expression prior to that of MHC expression in any cohort, causation may be clarified by a) targeting sections of the myoglobin promotor regions that are NOT known transcription factor binding motifs, and exploring if deleting or mutating them prevents the MHC –independent expression of myoglobin and b) deleting or mutating known NFAT and MEF binding motifs; if myoglobin expression ceases in these trials, experiments are warranted to test if there is another, upstream, calcineurin-independent but NFAT/MEF dependent pathway increasing the expression of these two factors. If myoglobin is expressed concurrently with MHC, but differentially in different lipid cohorts, these two experiments to clarify causation will still relevant, and any relevant promotor regions can be further tested by using a reporter construct to check promotor activity and myoglobin expression against the different lipid concentrations. Lastly, the effect of antimycotic on

myoglobin expression in varying lipid and oxygen conditions should be tested, to see if this accounts for the difference in results between this study and that of DeMiranda et al. (2012).

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CHAPTER 3 - CONSIDERATION OF DIET WAS NECESSARY FOR THE SUCCESSFUL ISOLATION AND CULTURE OF WEDDELL SEAL (*LEPTONYCHOTES WEDDELLII)* MYOBLASTS

Introduction

Weddell seals (*Leptonychotes weddellii*) and other pinnipeds have high myoglobin concentrations in their swimming muscles, ranging from 22.4 mg/g in a northern fur seal to 55.9 mg/g in the adult Weddell seal; for comparison, a cotton rat has 1.7 mg/g (Kanatous et al., 1999; Kanatous et al., 2008). This allows for a large oxygen store to draw from during dives, facilitating the use of lipid-based aerobic pathways, while decreasing anaerobic respiration and limiting lactate production (Kanatous et al., 2002). To further study the development of myoglobin regulation in a diving mammal, our lab developed a protocol to isolate and culture Weddell seal primary skeletal muscle cells, and determined media conditions for growth and differentiation by considering the seals' diet.

The diet of the Weddell seal depends on age, varies spatially and temporally, and can include pelagic and benthic fish, cephalopods, and crustaceans (Plotz, 1986; Burns et al., 1998; Lake et al. 2003). Lenky et al. (2012) examined the proximate composition of several species of nototheniid and myctophid fishes known to be Weddell seal prey. The pelagic *Pleuragramma antarcticum,* one of the seals' predominant prey species in the Weddell Sea, McMurdo Sound, Mawson Coast, the northern Vestfold Hills, and the Danco Coast of the Antarctic Penninsula, was found to have a high percentage of fat (7.2% wet mass), as were the myctophids *Electrona*

antarctica and *Gymnoscopelus nicholsi* (15.2 %, and 17.4% wet mass, respectively), which are consumed by Weddell seals along the Danco Coast and South Shetland Islands (Plotz, 1986; Casaux et al. 1997; Burns et al., 1998; Lake et al. 2003; Casaux et al. 2006; Lenky et al., 2012). Although *P. antarcticum* is the most common prey species in many locations, it should be noted that not all Weddell seal prey species are high in fat; the lipid composition of most of the benthic *Trematomus* species investigated by Lenky et al. (2012) ranged between 0.5%-4.6% of wet mass (Burns et al., 1998; Lake et al., 2003; Casaux et al., 2006).

The diet of the Weddell seal is reflected in the metabolic phenotype of their skeletal muscles, as determined by enzyme ratios. The citrate synthase (CS) to β-hydroxyacyl CoA dehydrogenase (HAD) ratio (CS:HAD), an indicator of how much the skeletal muscle relies on lipid versus carbohydrates to fuel metabolism, in both swimming and non-swimming skeletal muscle ranges between 0.1 and 0.4, suggesting a complete reliance on lipids for the generation of energy through aerobic metabolism (Reed et al., 1994; Kanatous et al., 2002; Kanatous et al., 2008). It is currently unknown if *in vivo* Weddell seal myotubes possess the same metabolic phenotype as the whole tissue from which they are derived, and/or if they have any degree of metabolic flexibility, that is to say, the preferential utilization of fat oxidation during fasting, with a switch to glucose in the presence of insulin, as described by Ukrpcova et al. (2005). If the seal cells share the metabolic phenotype of the muscle tissue, and are metabolically inflexible, we would expect them to need supplemental lipid to their growth and differentiation media.

We developed two methods that produced Weddell seal primary myoblasts, and, in doing so, found that myoblasts would not proliferate after initial plating with standard media. We

hypothesized that the growth and differentiation media of the myoblasts might require the addition of lipid, reflecting the high-fat diet of the Weddell seal. Based on the number of myotubes formed during differentiation, we conclude that the successful growth and differentiation media for seal cells is based on Dulbecco's modified Eagles media (DMEM), supplemented with 2.5%-5% lipid. Our results indicate that, when culturing non-model primary cells, researchers should consider the diet of the whole animal, and tailor the culture media accordingly. These seal primary skeletal muscle cells will aid researchers in understanding the molecular regulation of the unique adaptations to diving in Weddell seal skeletal muscle.

Materials and Methods

Weddell seal primary myoblast isolation

During the isolation of myoblasts from the Weddell seal, the researchers at McMurdo Station used two different myoblast isolation protocols based on the method of Pavlath (1996). Both isolation techniques yielded viable myoblasts, therefore we present both methods.

The Colorado State University ACUC has approved all protocols used in this study (IACUC 07-1641A-01), and samples were collected under permit from NMFS (MMPA # 10751788-00). Myoblasts were isolated from an adult seal, juvenile seal, and seal pup captured in McMurdo Sound, Antarctica in November 2006. The animal handling and biopsy protocol were performed as previously described; briefly, the seal was captured and kept calm using a head bag, and chemically immobilized with an IV injection of Telezol (1 mg kg^{-1}) (Kanatous et

al., 2008 and Trumble et al., 2010). A local injection of Lidocane (1 ml) was administered to the biopsy site, and the site was cleaned with Betadine. A small incision through the skin, blubber layer, and underlying fascia was made using a sterile #10 scalpel. The primary swimming muscle (*m. longissimus dorsi*) was biopsied with a sterile 6 mm biopsy cannula. Sterile forceps were used to transfer the muscle biopsy from the cannula. The biopsy was quickly disinfected in 70% ethanol and transferred into a 15 ml conical tube of Ham's F-10 media and placed on ice, where care was taken so that the sample did not freeze. The biopsy was then transported from the field site via snowmobile to the Albert P. Crary Science and Engineering Center at McMurdo Station; the traverse took 30 minutes.

Once at the Crary laboratory, the biopsies were processed in one of two ways; the first method is as follows: The biopsy was transferred to a 15 ml conical tube containing 0.4 ml of collagenase-D (Boehringer Mannheim, reconstituted to 10 mg/ml with PBS and 5 mM $CaCl_2$, product #1088-874) and dispase (Boehringer Mannheim, 2.4 u/ml, product # 295-825) per 100 mg of tissue. The biopsy was left to incubate with the added enzymes for 7 min at 37° C to digest connective tissue. The sample was then transferred to a sterile 6 well plate, where ~ 2 ml of HAM's F-10 growth media (Hyclone Laboratories, Logan, UT, USA) were added, and the biopsy was further minced using a razor blade. The plate was then placed in an incubator (37° C, 21% O_2, 5% CO_2) for 1 hour, after which the plate was removed from the incubator and observed for satellite cell migration under a microscope. Remaining pieces of tissue were then removed, and the plate was placed back in the incubator for 24 hours. The cells were checked daily and media was not removed from the dishes for 96 hours post-plating to ensure cells had a change to adhere to the bottom of the plate. After the 96 hours, when cells were observed growing a dish,

the media was changed daily. When the cells reached 40-60% confluence, they were passaged using trypsin. This method was tried on tissue from the adult male only, and successfully produced cells.

The second method used during isolation is as follows: The biopsy was removed from the media in which it was transported, and placed in a 60 mm cell culture dish, where it was minced into 1 mm pieces using a razor blade. The plate was placed into an incubator (37° C, 21% O_2, 5% CO_2) for 1 hour, after which the culture dishes were removed and observed. After 24 hours, the muscle tissue was removed from the plate. The cells were checked daily, and fresh Ham's F-10 growth media was added as needed. At day seven, media was changed from HAM's F-10 growth media to low glucose Dulbecco's modified Eagles media (DMEM) (Sigma Aldrich, St. Louis, MO, USA), 20% FBS, 1% penicillin/streptomycin antibiotic, and 1% sodium pyruvate (Gibco, Grand Island, NY, USA). At 13 days after initial sample plating, the cells (fibroblasts and myoblasts) were passaged to further isolate the myoblasts. The cells were seeded onto a 60 mm cell culture dish, after 30 minutes the supernatant containing the myoblasts was removed and placed onto a new cell culture dish leaving the majority of the fibroblasts behind. This method successfully produced cells from an adult, juvenile, and pup Weddell seal.

At the end of the field season in Antarctica (November, 2006), all cells isolated from both methods were frozen in liquid nitrogen and shipped to our laboratory at Colorado State University (CSU), Fort Collins, CO, USA where they are currently maintained and stored. At Colorado State University, only cells from the adult male were unfrozen, proliferated, and differentiated.

During the initial culture of the cells in Antarctica, low glucose DMEM was used in the growth media, and no lipid was added. In these cases, using normal or high glucose DMEM resulted in very poor to zero growth. When cells were unfrozen at our laboratory at CSU, they would not grow back in the low-glucose media. We thus considered the diet of the whole animal, and added lipid to the media. We found that myoblasts started to proliferate after adding at least 2.5% lipid (trials at 1% lipid produced very poor growth). After approximately 10 passages, cells began to do well in a high-glucose environment, as long as lipid was present.

Media recipes for Weddell seal skeletal muscle cells

Various growth and differentiation medias were made to test the potential effects on growth and differentiation of this unique primary cell line. The Weddell seal cells were grown and differentiated in their respective media supplemented with lipid throughout the experiment. Because our preliminary studies concluded that the cells did not grow in media without lipid supplementation, and grew poorly in media supplemented with 1% lipid, no "control" media with 0% lipid was tested in order to avoid wasting these rare samples, and lipid concentrations higher than 1% were assessed. A lipid mixture was supplemented to the culture media in concentrations of 2.5%, 5%, 7%, and 10% that were the same between the growth and differentiation media. The chemically defined mixture (Sigma Aldrich, St. Louis, MO, USA) is comprised of the following components: non-animal derived fatty acids (2 μg ml^{-1} arachadonic acid, and 10 μg ml^{-1} each of linoleic, linolenic, myristic, oleic, palmitic, and stearic acid), 0.22 mg ml^{-1} cholesterol from New Zealand sheep's wool, 2.2 mg ml^{-1} Tween-80, 70 μg ml^{-1} tocopherol acetate, and 100 mg ml^{-1} Pluronic F-68 solubilized in cell culture water. For the

differentiation media, human recombinant insulin (Gibco, Grand Island, NY, USA) was in a stock solution of 4 mg/ml and the bovine transferrin APO (Invitrogen, Carlsbad, CA, USA) was in a stock solution of 20 mg ml^{-1}. In this experiment, day one of differentiation was counted 24 hours after growth media was replaced by differentiation media.

Growth Media

Growth medias contained: 20% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, 1% sodium pyruvate, and either 2.5% , 5%, 7%, or 10% lipid mixture. The growth media was brought up to volume with high glucose DMEM, pH 7.6.

Differentiation Media

Differentiation medias contained: 2% Equine serum, 1% penicillin/streptomycin 10 µg/ml insulin, 10 µg/ml transferrin, and either 2.5%, 5%, 7%, or 10% lipid mixture. The differentiation media was brought up to volume with high glucose DMEM pH, 7.6.

Myotube count

After plating the cells on Petri dishes (100 x 20 mm), it took 15 days for enough Weddell seal skeletal muscle myoblasts to be grown for the experiment. During that time, the confluence (plate coverage) was estimated daily, and cells were passaged. It is important to note that not all lipid concentrations were passaged at the same time, as they did not reach appropriate confluence

at the same time (plates were passaged at 75%-100% confluence). For example at day 4, myoblasts in the 7% and 10% lipid media condition were not passaged with the rest of the lipid conditions, but instead passaged for the first time at day 8. Cells were monitored with a microscope and photographs were taken using a Canon Powershot (Canon Inc., Lake Success, New York, USA) mounted to a Carl Zeiss Invertoskope (Carl Zeiss Inc., Thornwood, New York, USA) at 100x magnification for the following seven days. Six plates of cells were grown in each lipid treatment, and each was photographed once per day for the seven days. A small circle was drawn onto the lid of each Petri dish with a Sharpie pen in an attempt to always photograph the same area of each plate; care was taken to not rotate the plate lid. The photographs were later analyzed with Carl Zeiss Axio Vision program to count the number of myotubes. The photo range had a field of view of 270 µm x 2037 µm. All 210 photos of the cells were analyzed. A 2 x 2 grid was created on the computer monitor to separate the photo into four 135 µm x 1018.5 µm boxes. This allowed myotube counts to be efficient and precise, and insured that each myotube was only counted once. The total number of myotubes were counted in each photograph and recorded.

Immunocytochemical confirmation of myotube presence

Plates were probed for desmin, and counterstained with DAPI to confirm the presence of multi-nucleated myotubes. Methods were based on Rosenblatt et al. (1995), with some modifications. Imaged cells were grown and differentiated on a 100 x 20 mm dish. All but 3 ml of media were removed from each plate, and replaced with 3 ml of a 1:1 mixture of ice-cold acetone and methanol. Samples were incubated at 4° C for 10 minutes, followed by three, 10-

minute washes in PBS. Plates were then incubated in 5% horse serum in PBS for 45 minutes to reduce non-specific binding. Mouse anti-desmin primary antibody (BioGenex) was diluted to 1:100 in 5% equine serum, incubated for 1.5 hours, and was followed by three 10 minute PBS washes. DAB chromogen was used for detection after a 1.5 hour incubation with 1:20 secondary antibody conjugated with horse-radish peroxidase. DAPI (300 nM) counter-stain was added for 10 minutes, after which images were taken.

Statistical Analysis

SigmaStat version 2.0 (Ashburn, VA, USA) was used to for all statistical analyses. Analysis of variance (one-way ANOVA) with Tukey *post-hoc* tests was used ($P \leq 0.05$), for comparison of myotubes numbers between lipid treatment groups each day. For the comparison of myotube counts between day 1 and day 7 within a lipid treatment group, a one-way repeated measures ANOVA with a Tukey *post-hoc* test was utilized for statistical analysis ($p \leq 0.05$). After day 4, only the 2.5%, 5.0%, and 7% groups were subject to ANOVA between group. For all calculations, if data did not pass normality or equal variance tests, ANOVA on ranks was performed. The results in all figures are presented as means ± s.e.m.

Results

Isolation and culture of primary Weddell seal myoblasts

We identified two methods that produced myoblasts capable of proliferation and differentiation (Figure 3.1). We were able to harvest cells from one adult, one juvenile, and one pup, but have, at this time, only differentiated cells from the adult male. One method utilized mechanical processing, while the second used both enzymatic and mechanical processing techniques. Samples were initially plated on HAM's F-10, but were moved to low-glucose DMEM-based media on day 7. Samples would only grow in low-glucose media until passage 10, when we were able to proliferate them in high glucose media. After being frozen down at -80° C with 100% isopropyl alcohol, and subsequently placed in liquid nitrogen for storage, thawed cells were capable of proliferating again only with the addition of lipid to the growth media.

Myotube count

After being thawed from the liquid nitrogen, Weddell seal cells did not proliferate in media unsupplemented with lipid, or supplemented with low (1%) amounts of lipid. When cultured in media supplemented with 2.5% and 5% lipid, the number of Weddell seal myotubes significantly increased between day 1 (24 hours after the initiation of differentiation) and day 7 (168 hours) of differentiation (n=6, $p=0.027$ and $p<0.001$ respectively; Figure. 3.2). Taking into account the

average myotube counts between days 1 and 7, the 2.5% lipid treatment group grew

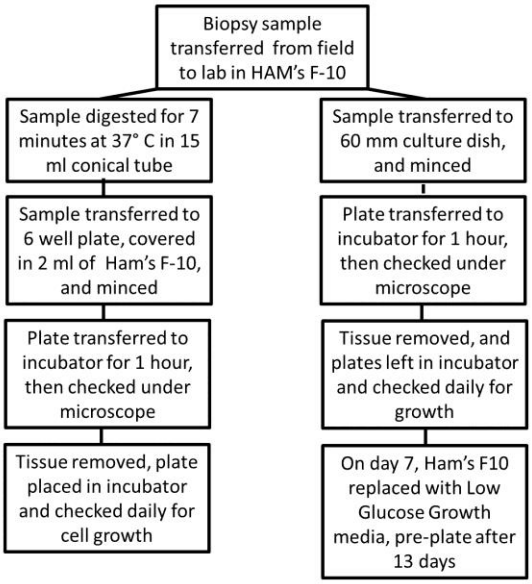


Figure 3.1 - Flow chart of two methods that produced primary Weddell seal skeletal muscle cells. One method (left) utilized mechanical and enzymatic processing methods, and the other (right) only relied on mechanical processing methods.

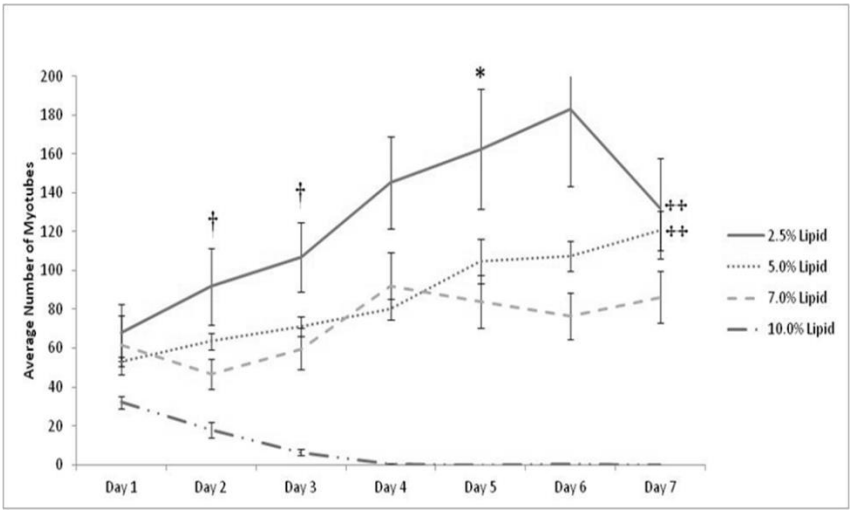


Figure 3.2 - Myotube counts from day one of differentiation to day seven. Significant differences between day one and day seven within one lipid treatment group indicated by ‡. Significant difference between 2.5% and 7% denoted by *. Significant differences between *both* 2.5 and 10 and 5.0 and 10 indicated by †. Significance between day one and day seven within lipid treatment groups was determined at $p \leq 0.05$, one way ANOVA with repeated measures, and significant difference between treatment groups for each day was determined by one way ANOVA. Data are presented as means ± s.e.m.

at a rate of 10.6 myotubes/day, the 5.0% group grew at 11.3 myotubes/day, the 7% group grew at 4.1 myotubes/day, and the 10% grew at -5.4 myotubes/day, and there was no statistical significance between the 2.5%, 5%, and 7% growth rates. All groups had statistically greater rates than the 10% treatment group, because almost all myotubes in that cohort died by day 4 of the trial ($p<0.05$). There was a significant difference between the 2.5% and 10%, and 5% and 10% treatment groups only on days 2 and 3 of differentiation ($p<0.05$ for both), as well as between 2.5% and 7% on day 5 of differentiation ($p=0.042$; Fig. 3.2). The presence of myotubes was confirmed via DAPI and Desmin staining (Fig. 3.3). Images of myotubes on days 2, 4, and 7 of differentiation can be found in Figure 4. Starting at day 1, the number of myotubes in 10% differentiation media decreased until zero myotubes were found on day 5, indicating high myotube mortality (Fig. 3.4). These results support the idea that high lipid content can be cytotoxic, although it is interesting to note that a high lipid concentration did not affect undifferentiated myoblast as dramatically.

Discussion

The cells supplemented with 2.5% and 5% lipid during proliferation and differentiation showed a significant increase in myotube number between the first (24 hours after the start of differentiation) and last day of differentiation. Given this result, we suggest that the most successful growth and differentiation medias for Weddell seal skeletal muscle cells contain between 2.5% and 5% lipid. It is interesting to note that the myotube count in the 2.5% media treatment groups dropped after day 6; a possible cause for this decrease is that these cells matured to the point of having functional contractile units earlier than the other treatments, as

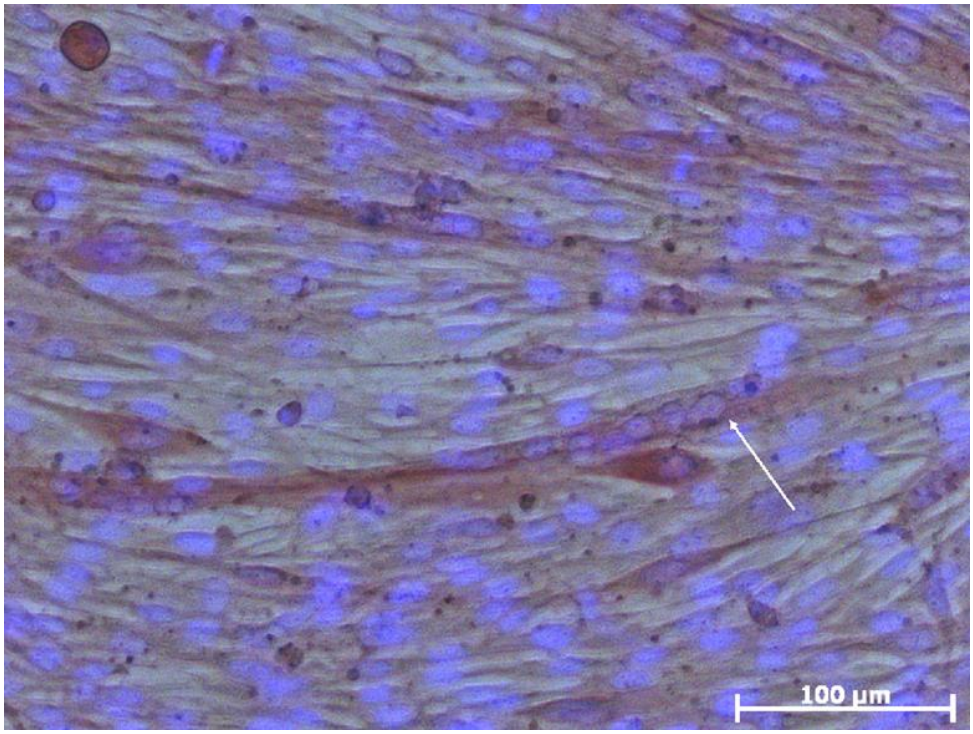
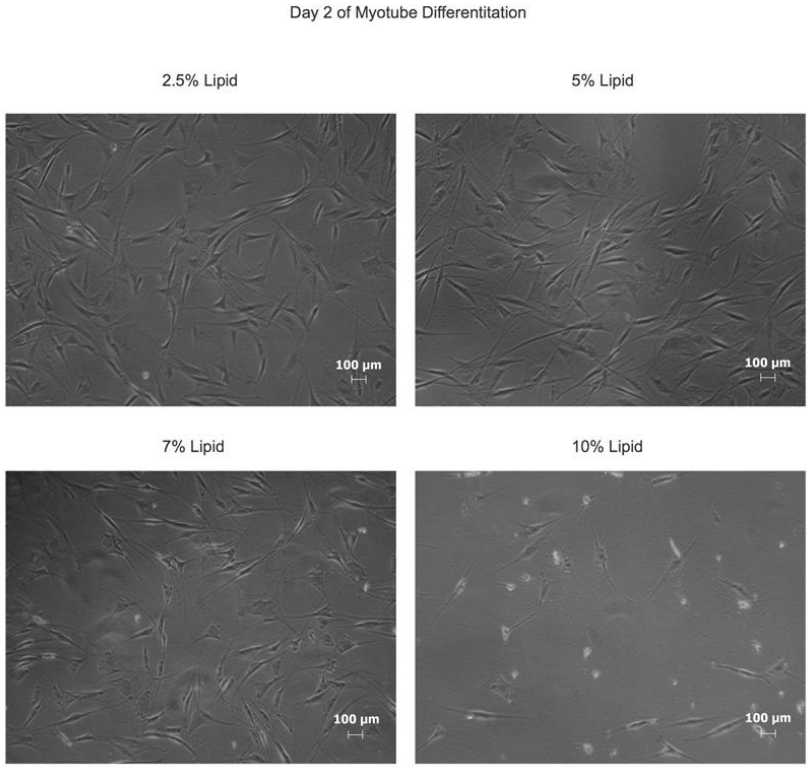


Figure 3.3 - Multinucleated myotubes were confirmed via anti-desmin antibody probe with chromogen and DAPI counter- staining. Arrow points to multinucleated myotube, 200x magnification.



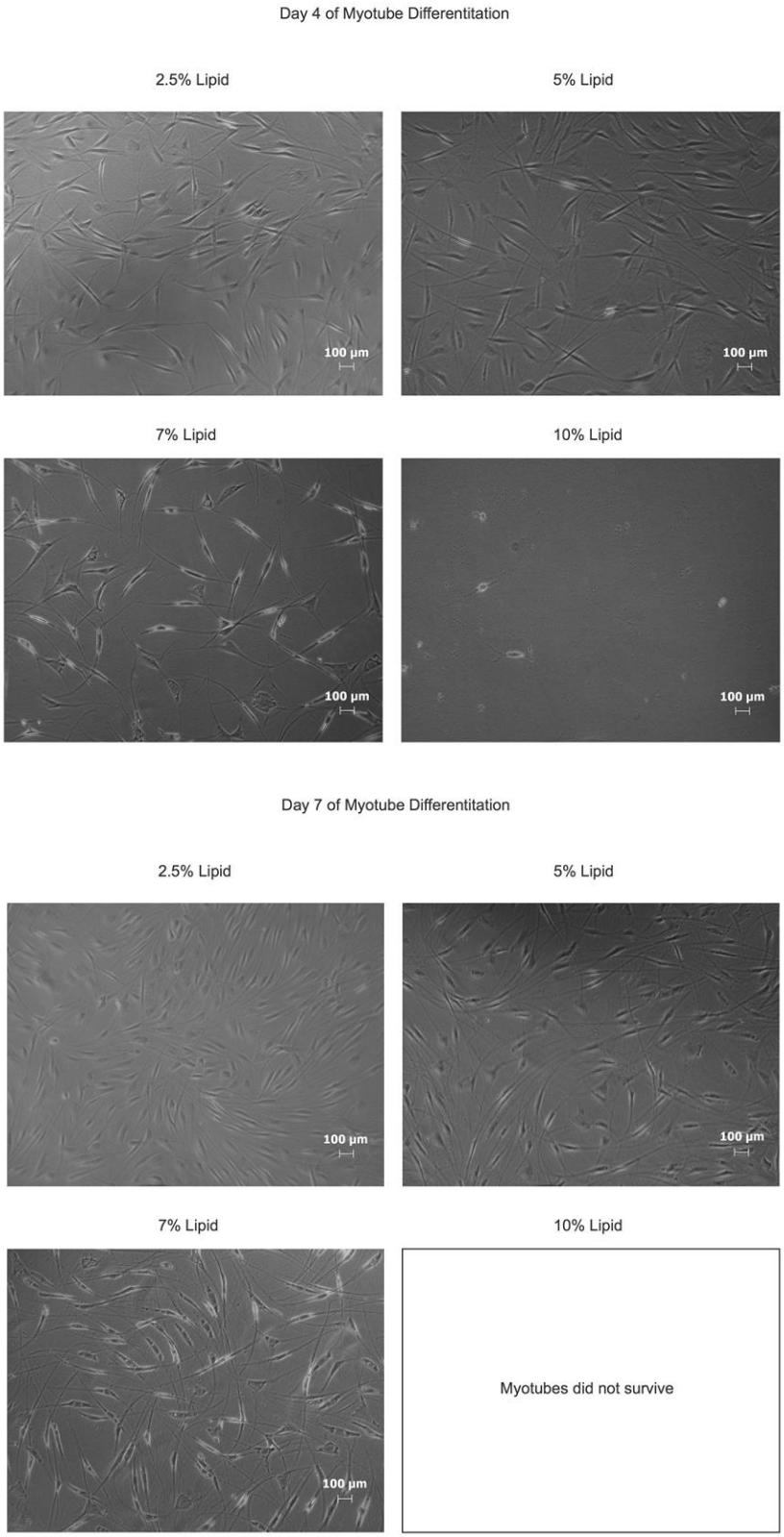


Figure 3.4 - Myotubes from each lipid treatment group on days 2, 4, and 7 of differentiation. Note the absence of an image for the 10% lipid mixture group. 100x magnification, Scale bars = 100 µm

contraction is one cause of myotube detachment in plates (Cooper et al., 2004). This suggests that experiments using Weddell seal cells in 2.5% media may need to consider harvesting earlier than day 6, or, to prolong a treatment, find a plating matrix that allows the matured fibers to contract without detachment, as in Cooper et. al. (2004), who sustained c2c12 myotubes atop a co-cultured layer of fibroblasts for 14 days after the initiation of differentiation.

Perhaps the most important result to report from our study is the fact that cultured Weddell seal skeletal muscle cells do not proliferate beyond initial plating and freezing without the addition of lipid. The primary culture of cells from various tissues has been successful in model animals, including mice and rats, using many types of growth and differentiation media. In primary cultures aimed at growing a specific cell type, the type of cell being grown has determined the media formulations, with little consideration for the type of species from which they were harvested. The cell type is important, because it dictates the nature of supplements needed in media, as well as other culture conditions, like the type of substrate or surface needed for cultivation. While this approach has been successful with cells from these model species, it is worth considering that the wild cousins of laboratory rodents are omnivores, and that the cells of these model species may have a more flexible metabolic phenotype because of their varied diet than the cells of the Weddell seal, an obligate carnivore. (National Research Council, 1995; Singleton and Krebs, 2007).

In the laboratory, the diet of the average rodent can range between 4.7-5% fat (wet weight, although these pelleted diets are low in moisture), with high-fat diets ranging between 18% -35% fat (pelleted diet calculated “as is”, ~3.5% moisture) (LabDiet, Certified Rodent Diet-

product #5002; Harlan Laboratories, Teklad Certified Rodent Diet- product #8728C; Harlan Laboratories, Teklad Custom Research Diet: Diet Induced Obesity Diets-product #TD.95217 and #TD.03584). It is not surprising that the percent of fat in the standard diet of these rodents is not much less than that of the favored Weddell seal prey *P. antarcticum,* and can ostensibly be higher. This flexibility seems to be shared by their cultured skeletal muscle cells; unpublished data from our lab have shown that immortalized mouse skeletal muscle cells (c2c12 ATCC, product #CRL-1772) can proliferate and differentiate in media with a range of glucose concentrations (1000 mg/L –4500 mg/L), and in the presence of between 2.5%-5% lipid (Schlater et al., unpublished). While the *in vitro* metabolic flexibility of c2c12 muscle cells in relation to that of *in vivo* mouse muscles has not been compared, studies by Ukropcova et al. (2005) in human skeletal muscle cells seem to suggest that there is a correlation between the *in vivo* metabolic flexibility of whole tissue, and *in vitro* measures of the metabolic flexibility of cultured cells. The authors found that, in healthy humans, the *in vitro* adaptability (the increase of fat oxidation with increase in high palmitate concentration) of skeletal muscle myotubes is correlated with the *in vivo* metabolic flexibility of the muscle from which they were cultured. However, comparisons of *in vitro* suppression, defined by the authors as the ability of glucose to suppress fat oxidization in the absence of insulin, was compared with an *in vivo* cohort subject to a euglycemic, hyperinsulinemic clamp, so direct comparisons of the ability to switch from fat oxidation to glucose are unclear. Additionally, Ukropcova et al. (2005) measured variability within one species, suggesting that broad statements about the relationship between whole tissue and cell muscle metabolic flexibility within a species cannot be made without first characterizing the intraspecies range of this relationship. Thus, while the metabolic phenotype and flexibility of skeletal muscle may have an intrinsic component, the nature of this contribution is uncertain.

It is possible that this lipid requirement of Weddell seal primary skeletal muscle cell culture is reflective of the lipid- based metabolic phenotype indicated by CS:HAD ratios in the whole muscle tissue from which the cells were biopsied, which, in turn, is reflective the seals' diets. Alternatively, it is also possible that the cells' lipid requirement is not imposed by a lipid-fueled aerobic metabolic phenotype, but is instead reflective of the role of lipid in regulatory myoglobin pathways. New evidence suggests that lipid either directly or indirectly increases the production of myoglobin in both c2c12 and Weddell seal skeletal muscle culture (De Miranda et al., 2012; Schlater et al., in review).

De Miranda et al. (2012) found that Weddell seal cells grown in normoxic conditions demonstrate increased myoglobin expression with higher lipid media concentrations (2.5% vs 5%). Prior to this result, increases in myoglobin were seen in the presence of hypoxia and a second stimulus, such as exercise, or after fiber-type changes (as reviewed in Kanatous and Mammen, 2010). In addition to its oxygen storage properties, myoglobin has the ability to scavenge reactive oxygen species (ROS). Schlater et al. (in review) found that the addition of a ROS scavenger to culture media supplemented with lipid reduces a lipid–induced myoglobin increase in normoxic c2c12 cells. Both of these results are suggestive of a yet uncharacterized lipid-based myoglobin regulatory pathway, and, in the case of the terrestrial c2c12 cells, one involved in ROS mitigation. It is possible that cultured Weddell seal skeletal muscle cells cannot proliferate without a lipid-stimulated expression of myoglobin; whether this would be due to the ROS scavenging or oxygen storage properties of myoglobin is unclear. Because the cells grown in normoxic environments exhibit this increase, a ROS-scavenging role in these cells is possible. Future studies in our laboratory will focus on unraveling the nature of these pathways.

Conclusions

The need for identifying the physiological requirements of the whole animal to optimize cell culture conditions may extend to other non-model organisms, and help establish new cell lines to help study the development of unique adaptations at the molecular level. If the need for lipid is related to diet, this culture protocol and media recipe may be extended to a variety of organisms that preferentially utilize lipids for energy production, including migratory birds and hibernators. Moreover, the consideration of a species' life-history may extend beyond diet, especially if it is distantly related to mammals, for which many commercial medias are developed. For example, the osmolarity of freshwater and marine mollusk hemolymph varies greatly from that of mammalian blood, and media osmolarity must be modified for successful primary culture (Yoshino et al., 2013). The present study represents a novel avenue to study the molecular regulation of the unique adaptations in skeletal muscles of diving mammals, and it is our hope that researchers will take our results and apply them towards other diving mammal species and more non-model organisms in the pursuit of understanding unique adaptations.

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CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

We have identified a need for the consideration of diet and other physiological requirements of the whole animal for the successful culture of cells from non-model organisms. In our case, muscle cells cultured from an animal with a high fat diet required lipid supplementation of 2.5%-5% of total volume to both growth and differentiation media. Using consistent myoblast proliferation and myotube production as metrics, the culture of these cells has been thus far successful, however, it is possible that the culture media could be further optimized with the addition of other supplements, such as chick embryo extract, and with the definition of the optimal lipid concentration within the 2.5%-5% range. Moreover, the addition of a more suitable plating matrix, such as fibroblasts, may allow for longer differentiation periods and produce more homogenous and mature myotubes. Future studies should be directed at optimizing both culture and plating conditions.

The need for future studies aimed at producing mature myotubes is further warranted by our findings that cultured c2c12 and seal skeletal muscle cells exhibit minimal amounts of adult MHC isoforms if differentiated for 7 days under the media and plating conditions outlined in chapter 2. Producing mature myotubes would more definitively elucidate *in vivo* mechanisms driving the production of large myoglobin stores in the Weddell seal swimming muscles. Our studies of what turned out to be immature myotubes may support the idea that, in cultured cells, there is a mechanism independent of MHC isoform, or hypoxia and a second stimulus, regulating myoglobin production. Other studies suggest that these mechanisms may be driven by lipid or lipid metabolites, and may be involved in myoglobin's role as a ROS scavenger (Schlater et al. *in*

review). On the other hand, we only measured one time point for MHC and myoglobin expression, and thus is possible that MHC expression preceeded myoglobin expression, and the myoglobin expression is low because MHC expression is low. Future studies should include a high-temporal resolution analysis of RNA and protein expression in cultured myotubes, as well as a search for novel transcription factor binding motifs in the myoglobin promoter and potential calcineurin-independent but NFAT/MEF dependent pathways.