THESIS

IN THE FACE OF HYPOXIA: MYOGLOBIN EXPRESSION UNDER HYPOXIC CONDTIONS IN CULTURED WEDDELL SEAL SKELETAL MUSCLE CELLS

Submitted by

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ABSTRACT

IN THE FACE OF HYPOXIA: MYOGLOBIN EXPRESSION UNDER HYPOXIC CONDITIONS IN CULTURED WEDDELL SEAL SKELETAL MUSCLE CELLS

The hallmark adaptation to breath-hold diving in Weddell seals (*Leptonychotes weddellii*) is enhanced concentrations of myoglobin in their skeletal muscles. Myoglobin is a cytoplasmic hemoprotein that stores oxygen for use in aerobic metabolism throughout the dive duration. In addition, throughout the duration of the dive, Weddell seals rely on oxygen stored in myoglobin to sustain aerobic metabolism in which lipid is the primary contributor of acetyl CoA for the citric acid cycle. Together, enhanced myoglobin concentrations and a lipid-based aerobic metabolism represent some of the unique adaptations to diving found in skeletal muscle of Weddell seals. This thesis presents data that suggests cultured Weddell seal skeletal muscle cells inherently possess adaptations to diving such as increased myoglobin concentrations, and rely on lipids to fuel aerobic metabolism. I developed the optimum culture media for this unique primary cell line based on myoblast confluence, myoblast growth rates, myotube counts, and myotube widths. Once the culture media was established, I then determined the *de novo* expression of myoglobin under normoxic and hypoxic oxygen conditions and the metabolic profile of the myotubes under each oxygen condition. I found that the optimum culture media for the Weddell seal primary skeletal muscle cells high glucose Dulbecco's modified eagles media (DMEM) supplemented with a lipid mixture at a final concentration of 2.5%, based on myoblast confluence, myotube counts, and myotube widths. I also determined that the Weddell seal skeletal muscle cells increased myoglobin under hypoxia, to levels greater than a C_2C_{12} control cell line, which is in direct contrast to previous studies using terrestrial mouse models. While the

Weddell seal cells increased myoglobin, the metabolic enzymes responded similarly to the control cell line under both oxygen conditions. In addition, I found that increasing the concentration of lipid in the culture media increased myoglobin under normoxic conditions. To our knowledge, these studies represent the first successful isolation and culture of primary skeletal muscle cells from a diving mammal and the first metabolic profile and myoglobin expression measurements under varying oxygen conditions. This unique primary cell line and my preliminary data will enable future researchers to investigate the molecular regulation of the unique adaptations in seal skeletal muscle and unravel the elusive regulatory pathways of myoglobin expression in diving mammals. Understanding the regulatory mechanisms of an oxygen storage protein will have profound impacts on various human diseases that include tissue hypoxia and ischemia.

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DEDICATION

I would like to dedicate this thesis to my fiancé Briana Trout, for all of the love and support she has given me throughout the years.

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CHAPTER 1: Introduction

Myoglobin is a cytoplasmic hemoprotein found in cardiac and skeletal muscle and is able to bind reversibly bind oxygen. Due to the location of myoglobin protein, in the cytoplasm of the cell, the oxygen bound to myoglobin is able to diffuse to locations in the cell where oxygen is needed (Garry et al., 2003; Wittenberg and Wittenberg, 2003; Wittenberg et al., 1975). The regulation of myoglobin has been extensively studied using skeletal muscle tissue from mice (*Mus musculus*) and skeletal muscle cell lines (C_2C_{12}). Previous studies have shown the calcineurin/ nuclear factor of activated T-cells (NFAT) pathway is the primary mode to regulate the expression of myoglobin in skeletal muscle (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009). As skeletal muscle contracts, calcium is released from the sarcoplasmic reticulum in order to allow myosin/actin interaction. The released calcium activates the calcium-dependent calcineurin enzyme, which upon activation dephosphorylates NFAT, which translocates into the nucleus and activates target genes including myoglobin (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009; Rao et al., 1997). These studies implicate calcium, from skeletal muscle contraction, as the essential stimuli for myoglobin expression in terrestrial models.

Historically, myoglobin has been studied in hypoxia-adapted humans and animals in an effort to understand the role myoglobin plays in low oxygen conditions. In 1962, Balthazar Reynafarje studied skeletal muscle adaptations in Peruvian miners living at high altitude. In this key study, he found distinct changes in the oxidative capacity of muscle in the legs of the subjects, which included a 16% increase in myoglobin when compared to control subjects living at lower altitudes (Reynafarje, 1962; Hoppeler et al., 2003). This early study introduced the idea

of oxidative changes in skeletal muscle, especially increasing myoglobin protein expression, in response to environmental hypoxia (Hoppeler et al., 2003). This major finding stood for more than 25 years until a review was published examining data from environmental hypoxia experiments on animals. After a review of the then current literature, Banchero (1987) concluded that environmental hypoxia alone was not sufficient to drive skeletal muscle changes, which include increasing oxidative enzymes and myoglobin concentrations, to adapt to hypoxic environmental conditions. Banchero (1987) proposed that hypoxia alone was not sufficient to cause positive adaptive changes in skeletal muscle, but rather a combination of cold or exercise was needed (Banchero, 1987; Hoppeler et al., 2003). It was more likely that activity level of the test subjects was ignored in Reynafarje's 1962 study, most likely because he was unaware of the effect of exercise on oxidative enzyme changes (Hoppeler et al., 2003). The relationship between exercise, activity, increasing mitochondrial oxidative enzymes and myoglobin concentrations was not studied until the late sixties, when Holloszy and colleagues showed increases in oxidative enzymes in Winstar rats (*Rattus norvegicus*) after a 12 week exercise protocol (Holloszy, 1967).

Myoglobin expression under normoxic oxygen conditions and exercise has been a source of conflicting results and controversy. The Holloszy laboratory, showed that myoglobin in the quadriceps of exercised Carworth's rats increased when compared to the sedentary rat group, after 15 weeks of exercise training. They concluded that prolonged exercise was sufficient to increase myoglobin in only the working locomotory muscles of the exercised rats (Pattengale and Holloszy, 1967). It is interesting to note that Holloszy hypothesized that myoglobin levels in the rat's skeletal muscles have reached their physiological limits. The hypothesis proposed by Holloszy and colleagues leaves the possibility open for secondary factors to be involved in

further increases in myoglobin expression, which includes genetic factors and skeletal muscle contraction (Pattengale and Holloszy, 1967). However, a few studies have shown that despite high intensity aerobic exercise and resistance training, myoglobin was not increased in working skeletal muscles, which conflicted with previous experiments (Harms and Hickson, 1983; Masuda et al., 1999). In addition, experiments examining myoglobin regulation under normoxic conditions using mouse cell lines (C_2C_{12}) and whole mouse models have shown that simulated exercise was not sufficient to increase myoglobin expression when compared to non-exercise control groups (Kanatous et al., 2009). Kanatous and colleagues (2009) also showed that skeletal muscle contraction under normoxia resulted in a selective release of calcium from the sarcoplasmic reticulum activated the calcineurin/NFAT pathway to target myoglobin gene expression (Kanatous et al., 2009).

Myoglobin expression under environmental hypoxia has been recently studied extensively using mouse skeletal muscle cells culture, whole mouse models, and human exercise subjects. Studies using human subjects exercised under simulated hypoxic conditions showed increases in myoglobin mRNA transcripts in the working skeletal muscle (Hoppeler and Vogt, 2001; Vogt et al., 2001). In an elegantly designed study, it was shown that myoglobin increases beyond control concentrations when environmental hypoxia is coupled with skeletal muscle contraction (Kanatous et al., 2009). When the C_2C_{12} mouse skeletal muscle cells were cultured under hypoxia, with no artificial stimulation to contract, myoglobin actually decreased. The study determined hypoxia altered calcium release from the endoplasmic reticulum, which prevented NFAT from translocating to the nucleus, thus preventing myoglobin gene expression (Kanatous et al., 2009). However, when the mouse skeletal muscle cells were stimulated to contract under hypoxia with extracellular calcium, the result was a significant increase in

myoglobin protein expression, to levels significantly greater than myoglobin protein expression measured in mouse cells cultured in normoxic oxygen conditions (Kanatous et al., 2009). This mouse study coupled with human exercise studies has implicated the need for a secondary stimulus (exercise or contraction) in order for skeletal muscle to significantly increase myoglobin concentrations under environmental hypoxia. Although the regulatory mechanism of myoglobin expression has been extensively studied using terrestrial models (mouse), the pathways have not been studied in diving mammals, which have been shown to have extremely high myoglobin concentrations when compared to athletic terrestrial mammals such as greyhounds and horses. Diving mammals may possess unique pathways and unknown regulatory mechanisms that allow them to express and maintain high concentrations of myoglobin.

Weddell seals (*Leptonychotes weddellii* Lesson, 1826) are air breathing diving mammals that overcome periods of tissue hypoxia and ischemia during the duration of a breath-hold dive. Upon diving, which is a period of high skeletal muscle activity levels, Weddell seals exhibit a dive response in water that is in direct contrast with terrestrial exercise responses on land. The Weddell seal dive response includes a cessation of ventilation, extreme bradycardia, and peripheral vasoconstriction to the working skeletal muscle, while the exercise response in terrestrial mammals on land consists of increasing ventilation, tachycardia, and peripheral vasodilation to the working skeletal muscle. Because diving is a highly active period for seals they must be able to maintain skeletal muscle function to engage in foraging activities. Weddell seals are able to maintain skeletal muscle function despite increasing ischemia and subsequent increasing tissue hypoxia in part because of unique adaptations in their skeletal muscle. Kanatous and colleagues (2002 and 2008) showed, using muscle biopsies from the primary swimming muscle (*M. longissimus dorsi*), that Weddell seals to have a high reliance on lipid-based aerobic

metabolism, mitochondrial volume densities similar to sedentary terrestrial mammals, increased oxygen storage capacities and diffusion capacities, and a reduced dependence on blood borne oxygen (Kanatous et al., 2002; Kanatous et al., 2008). Together, the skeletal muscle adaptations allow Weddell seals to engage in breath-hold dives for long periods of time while maintaining skeletal muscle function.

The hallmark adaptation found in diving mammals is enhanced concentrations of myoglobin in their skeletal muscle, when compared to terrestrial mammals. Previous studies have measured myoglobin to be up to 10-fold greater in the primary swimming muscles when compared to athletic terrestrial mammals including dogs and ponies (Hochachka and Foreman, 1993; Kanatous et al., 2002; Reed et al., 1994). Adult Weddell seals have myoglobin concentrations in their primary swimming muscles to range from 45.9 ± 3.3 to 55.9 ± 2.9 mg myoglobin g^{-1} wet muscle mass (Kanatous et al., 2002; Kanatous et al., 2008; Noren et al., 2005). Weddell seal pups, aged 3-5 weeks, possess enhanced concentrations of myoglobin in their skeletal muscle, which are about 35 mg myoglobin g^{-1} wet muscle mass. This amount of myoglobin early in the life a Weddell seal pup is even more impressive when compared to other adult marine mammals of different species. Adult Stellar sea lions (*Eumetopias jubatus*) and Northern fur seals (*Callorhinus ursinus*) have roughly 28.7 ± 1.5 and 22.4 ± 2.5 mg myoglobin g^{-1} wet muscle mass, respectively in their primary swimming muscle (*Pectoralis major*) (Kanatous et al., 1999; Kanatous et al., 2008). In contrast to the adult divers, the Weddell seal pups during a 3-5 week nursing period are considered to be non-diving as they are on the ice in close proximity to their mother. Additionally, during the nursing period, Weddell seal pup's only source of dietary intake is milk from the mother, as they are not yet diving and foraging independently (Reijnders et al., 1990). During the nursing period Weddell seal pups are not engaging in breath-

hold diving, so their primary swimming muscles (*longissimus dorsi*) are not receiving the normal cues associated with prolonged skeletal muscle activity and hypoxia. Without the skeletal muscle activity in the primary swimming muscles, the calcium signaling pathways associated with myoglobin gene expression are not activated. However, during this time, Weddell seal pups possess high myoglobin concentrations that are 63% of the amount measured in adult swimming muscles (Kanatous et al., 2008).

Studies utilizing terrestrial mammal models have implicated the calcium/calcineurin pathway, through skeletal muscle contraction, as the regulatory mechanism for myoglobin expression. In addition, the need for as secondary stimulus is required to enhance myoglobin expression to levels beyond normal values. Interestingly it appears that Weddell seal pups express high myoglobin concentrations before experiencing the physiological cues associated with the myoglobin regulatory pathway. Diving mammal pups appear to inherently possess an ability to enhance myoglobin concentrations to levels well above terrestrial mammals before experiencing significant skeletal muscle activity associated with diving. Understanding the *de novo* metabolic properties and myoglobin concentrations of developing Weddell seal skeletal muscle cells may help elucidate the unique regulatory pathways that allow elite divers to develop great internal myoglobin concentrations. To understand the expression of myoglobin of developing Weddell seal skeletal muscle cells, cultured Weddell seal myoblasts were utilized for the studies presented in this thesis.

Skeletal muscle tissue contains quiescent satellite cells, or myoblasts, around the periphery of the muscle fiber (Mauro, 1961). Myoblasts are undifferentiated skeletal muscle cells that do not possess the structural and contractile characteristics associated with functional myotubes. Within the muscle tissue, myoblasts are able to proliferate and migrate to sites of

skeletal muscle injury, which upon receiving myogenic signals, fuse together to form new multinucleated muscle fibers. The functional muscle fibers, named myotubes in culture, are not able to proliferate, but retain the ability to contract and express the same proteins, such as myoglobin, as muscle tissue (Scharner and Zammit, 2011). Because myoblasts are not fully differentiated, they can be isolated from skeletal muscle tissue and cultured to study the early stages of skeletal muscle development. Differentiating myoblasts into functional myotubes in culture reflect similar myogenic regulatory factors found in true muscle development (Hepple, 2006). This unique feature of differentiation in cultured skeletal muscle cells may help decipher the regulatory pathways by which Weddell seals up-regulate myoglobin expression while providing a novel way to study myoglobin expression in elite diving mammals.

The purpose of the present studies is to develop a cell culture growth protocol and differentiation media for a novel Weddell seal skeletal muscle cell line, then investigate myoglobin concentrations and the metabolic profile of the cultured cells to determine if Weddell seal skeletal muscle cells are inherently adapted to possess high myoglobin concentrations. To accomplish this goal, I tested various culture media supplemented with varying amounts of a lipid mixture. Based on myoblast growth rates, the number of myotubes after differentiation, and overall size of differentiated myotubes, I determined the optimum media recipe for a novel Weddell seal skeletal muscle cell line isolated from a muscle biopsy of an adult Weddell seal. Then, I examined non-stimulated Weddell seal skeletal muscle cells under a normoxic (21% O_2) with a PO_2 of 159 mmHg condition, and a hypoxic (0.5% O_2) with a PO_2 of 38 mmHg culture condition. I measured myoglobin concentrations and metabolic enzyme activities of the cells after seven days of differentiation into myotubes. The enzymes assayed included: citrate synthase (CS), the enzyme in the first step of the citric acid cycle and an indicator of aerobic

capacity, lactate dehydrogenase (LDH), the enzyme responsible for the conversion of pyruvate to lactate and an indicator of anaerobic capacity, and β-hydroxyacyl CoA dehydrogenase (HAD), an indicator of β-oxidation of fatty acids. In this second experiment, I also tested the effects of varying lipid concentrations on myoglobin expression in the cultured Weddell seal skeletal muscle cells to determine if lipids play a role in myoglobin expression. The concentration of the lipid mixture supplemented to the growth and differentiation media was comprised of 50% saturated fatty acids, 33.5% polyunsaturated fatty acids, and 16.5% monounsaturated fatty acids. The specific components were 3 different polyunsaturated fatty acids, 2 different monounsaturated fatty acids, and 1 saturated fatty acid. I hypothesized that the Weddell seal skeletal muscle cells would require lipids supplemented to the growth and differentiation media due to the Weddell seal's reliance on lipids as a primary source of energy. I also hypothesized that the Weddell seal skeletal muscle cells will have high concentrations of myoglobin *de novo* that reflect the concentrations of myoglobin observed in tissue. In addition I hypothesized that β-hydroxyacyl CoA dehydrogenase activity would increase in response to the increasing amounts of lipid supplemented in the growth media. I further hypothesized that seal cells will respond to environmental hypoxia similarly to the terrestrial mammalian cell line (C_2C_{12} cells) in that citrate synthase enzyme activity and myoglobin concentration will remain the same or decrease under hypoxia and lactate dehydrogenase activity will increase under hypoxia.

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CHAPTER 2: The isolation and culture of Weddell seal (*Leptonychotes weddellii*) myoblasts and the effects of varying lipid concentrations on myotube morphology

Summary

Skeletal muscle cells contain a population of undifferentiated satellite cells located around the periphery, which can be isolated and cultured to examine molecular adaptations of skeletal muscle. Weddell seals exhibit unique skeletal muscle adaptations allowing them to maintain skeletal muscle function despite increasing hypoxia associated with long duration breath-hold diving. To understand the molecular adaptations to hypoxia of Weddell seal skeletal muscle, we developed an isolation and culture technique for Weddell seal myoblasts. We hypothesized isolated Weddell seal cells would require lipid supplementation to growth and differentiation media, which is reflective of their diet. We tested the addition of various lipid concentrations to the culture media, and measured overall myoblast confluence, the number of myotubes formed during differentiation, and the widths of the formed myotubes. Based on the measured criteria, we determined the best growth and differentiation media for Weddell seal skeletal muscle cells consists of high glucose DMEM supplemented with a total lipid concentration of 2.5%. The methods and media conditions presented in this study greatly enhance the potential for researchers to culture skeletal muscle cells from other non-model organisms, while providing new avenues for research techniques to investigate molecular regulatory pathways driving adaptations seen in the whole animal.

Introduction

Skeletal muscle fibers contain quiescent satellite cells located around the periphery between the basal lamina and sarcolemma (Mauro, 1961). These satellite cells, known as myoblasts, represent a population of undifferentiated stem cells able to migrate to sites of skeletal muscle injury, or become activated to fuse together and create new multinucleated muscle fibers. Culturing skeletal muscle myoblasts and differentiating myoblasts into functional myotubes, allows researchers to examine myogenic regulatory factors reflective of true muscle development (Hepple, 2006). By examining regulatory factors in culture conditions, researchers can decipher molecular regulation programs that drive mature tissue phenotypes, and specific skeletal muscle adaptations.

Like many marine mammal species, Weddell seals (*Leptonychotes weddellii* Lesson 1826) routinely overcome the harmful effects of hypoxia and ischemia throughout a breath-hold dive. Weddell seals maintain skeletal muscle function throughout the dive despite increasing tissue hypoxia, which is an otherwise pathological condition, in part because of several unique skeletal muscle adaptations. A hallmark adaptation in Weddell seals, and other diving mammals, are large myoglobin concentrations in skeletal muscle, which store large amounts of oxygen to use while diving, decreasing anaerobic respiration thus limiting lactate production. Another adaptation Weddell seals possess is the ability to maintain a reliance on fatty acid oxidation for energy production under hypoxic conditions. Studies have measured citrate synthase to β-hydroxyacyl CoA dehydrogenase ratios between 0.2 and 0.4 (Kanatous et al., 2002; Kanatous et al., 2008), suggesting a complete reliance on lipids for the generation of energy through aerobic metabolism.

To further study the development of these adaptations at the cellular level, and elucidate the molecular regulation of myoglobin in a diving mammal, our lab developed a protocol to isolate and culture Weddell seal primary skeletal muscle cells. Our laboratory successfully isolated and cultured primary skeletal muscle cells from a Weddell seal, and determined the optimum media conditions for growth and differentiation. We found the best media for this unique cell line is high glucose DMEM (4,500 mg l^{-1}) supplemented with 2.5% lipid, based on overall growth, myotube size, and number of myotubes formed. Our results indicate to culture this unique primary cell line, researchers must take into account the physiology of the whole animal, and tailor the culture media accordingly. The Weddell seal primary skeletal muscle cell line 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

To isolate the myoblasts from Weddell seal skeletal muscle tissue, two different isolation protocols were used in order to determine if one resulted in the greatest number of viable cells. Both isolation techniques yielded viable myoblasts therefore we present both methods which may be used in combination or separate from each other.

The Colorado State ACUC has approved all protocols used in this study (IACUC #), and samples were collected under permit from NMFS (MMPA # 10751788-00). Weddell seal myoblasts were isolated from an adult male (430 kg) Weddell seal captured in McMurdo Sound,

Antarctica in November 2006. The animal handling and biopsy protocol were performed as previously described (Kanatous et al., 2008 and Trumble et al., 2010). In brief, the seal was captured using a head bag, and chemically immobilized with an IV injection of telezol (1 mg kg^{-1}). 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 (*longissimus dorsi*) was biopsied with a sterile 6mm 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. 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 between 30 and 80 minutes.

The method detailed below is as previously described (De Miranda Jr. et al., 2012). In brief, the muscle biopsy was briefly dipped into 70% ethanol and placed into Hams F-10 growth media (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and 1% penicillin/streptomycin antibiotic (Sigma Aldrich, St. Louis, MO, USA), and taken to the Crary Laboratory. The biopsy was then placed in a sterile 60 mm cell culture dish and minced with a sterile razor blade into 1 mm pieces. The pieces of muscle tissue were placed into a new 60 mm cell culture dish and 3 ml of HAM's F-10 growth media was added to the plate. The plate was placed into a humidified incubator set to 37° C, 21% O_2, and 5% CO_2. The plate was monitored for myoblast migration for six days. Fresh Ham's F-10 growth media was added as needed. The cells were then passaged in order to enrich the myoblast cell type. The cells were seeded on a new 60 mm culture dish,

after 30 minutes the supernatant containing myoblasts were removed and placed onto a fresh 60 mm culture dish, leaving the fibroblasts behind.

A 2nd muscle biopsy was quickly rinsed in 70% ethanol and placed in a 50 ml conical tube that contained sterile phosphate buffered saline (PBS). In the lab, the biopsy was placed in a sterile 60 mm cell culture dish and minced with a sterile razor blade into 1 mm pieces. The pieces of muscle tissue were placed into a new 60 mm cell culture dish and 3 ml of HAM's F-10 growth media (Hyclone Laboratories, Logan, UT, USA), containing 20% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA) and 1% penicillin/streptomycin (Hyclone Inc., Logan, UT, USA) was added to the plate. The plate was placed into a humidified incubator set to 37° C and atmospheric conditions of 21% O_2, and 5% CO_2. After an hour in the incubator, the plate was removed to inspect for myoblast migration. After 24 hours the tissue pieces were removed, leaving the migrated myoblasts and fibroblasts on the plate. Media was changed as needed over the course of six days. At day seven, media was changed from HAM's F-10 growth media to high 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. 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, Fort Collins, CO, USA where they are currently maintained and stored.

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. 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 lipid mixture was purchased from Sigma Aldrich product #: L0288 (Sigma Aldrich, St. Louis, MO, USA). The chemically defined mixture 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. The overall ratio of the fatty acid composition was 50% saturadted fatty acids, 33.5% polyunsaturated fatty acids, and 16.5% monounsaturated fatty acids. 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 1 of differentiation was counted 24 hours after growth media was replaced by differentiation media. The following is list of growth and differentiation medias used during this experiment:

Growth Media

High glucose DMEM with 2.5% lipid supplementation: 20% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 2.5% lipid mixture, 75.5% high glucose DMEM pH 7.6.

High glucose DMEM with 5% lipid supplementation: 20% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 5% lipid mixture, 73% high glucose DMEM pH 7.6.

High glucose DMEM with 7% lipid supplementation: 20% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 7% lipid mixture, 71% high glucose DMEM pH 7.6.

High glucose DMEM with 10% lipid supplementation: 20% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 10% lipid mixture, 68% high glucose DMEM pH 7.6.

Differentiation Media

High glucose DMEM with 2.5% lipid supplementation: 2% Equine serum, 10 μg/ml insulin, 10 μg/ml transferrin, 2.5% lipid mixture, 75.5% high glucose DMEM pH 7.6.

High glucose DMEM with 5% lipid supplementation: 2% Equine serum, 10 μg/ml insulin, 10 μg/ml transferrin, 5% lipid mixture, 73% high glucose DMEM pH 7.6.

High glucose DMEM with 7% lipid supplementation: 2% Equine serum, 10 μg/ml insulin, 10 μg/ml transferrin, 7% lipid mixture, 71% high glucose DMEM pH 7.6.

High glucose DMEM with 10% lipid supplementation: 2% Equine serum, 10 μg/ml insulin, 10 μg/ml transferrin, 10% lipid mixture, 68% high glucose DMEM pH 7.6.

Pre-differentiation myoblast confluence

Pre-differentiation confluence was determined for each media condition during the growth phases of myoblasts. The confluence was interpreted as the percentage of a 100 mm cell culture dish that was covered by Weddell seal myoblasts. Confluence was determined during the entire growth period and continued until the day the cells were differentiated into myotubes. The days recorded include day 2 after initial myoblast plating, day 4 at first passage after initial plating, day 8, day 9, day 11 at second passage after initial plating and the final record was made at day 15 when differentiation media was added to the myoblasts. It is important to note that not all lipid concentrations were passaged at the same time. 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.

Myotube count

After plating the cells on culture 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 recorded daily. Cells were passaged on day 4 and day 11, and then differentiated on day 15. Cells were monitored with a microscope and photographs were taken using a Canon Powershot A620 (Canon Inc., Lake Success, New York, USA) mounted to a Carl Zeiss Invertoskope (Carl Zeiss Inc., Thornwood, New York, USA) using a 10x objective lens for the following 7 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 culture dish with a sharpie pen in an attempt to always photograph the same area of each plate. 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.

Myotube morphology measurements

Multiple photographs were taken each day of a seven-day period after the differentiation of the myoblasts using a Canon Powershot A620 mounted to a Carl Zeiss Invertoskope with a 10x objective lens. All photographs were taken in a different location on the cell culture dish for each day and were randomly analyzed using Zeiss Axio Vision software. Using the photographs, for each day of differentiation, the average width of the myotubes was determined. The myotubes on each photograph taken were measured using the measuring tool on the Zeiss Axio Vision software program. The myotubes were measured by width of the approximation of the middle of the cell. Cell width indicates the measurement of the myotube at the approximate center of the myotube from one side of the cell membrane to the membrane on the opposite side. Each measurement indicates one myotube width and each photograph taken had 20 measurements. The width measurements were recorded in a table created by the Zeiss Axio Vision software and the data from each photograph was pooled together and analyzed.

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 the myotube width measurements and the results in all figures are presented as means ± s.e.m. For the myotube count data, a one-way repeated measures ANOVA with a Tukey *post-hoc* test was utilized for statistical analysis because we measured the same cell culture dishes during the duration of the experiment. For the one-way repeated measures ANOVA significance was determined at ($P \leq 0.05$).

Results

Pre-differentiation myoblast confluence

On day 2 after initial plating, the Weddell seal myoblasts grown in 2.5% and 7% lipid were at 45% confluence. On day 2 the myoblasts grown in 5% and 10% were at 20% and 15% confluence, respectively. At day 4 the 2.5% and 5% lipid myoblasts were at 80% confluence and were passaged, while the myoblasts grown in 7% and 10% lipid were at 40% and 35% confluence, respectively, and were not passaged. At day 8, the myoblasts grown in 2.5% and 5% were both at 60% confluence. The myoblasts grown in 7% and 10% lipid grew slower than the 2.5% and 5% lipid conditions, and remained at 40% and 35% respectively, the same confluence as day 4. At day 9, the myoblasts grown in 2.5% and 5% lipid were confluent at of 80% and 75% respectively. Interestingly, at day 9, the myoblasts grown in 7% and 10% increased their confluence to 65% and 80%, respectively. At day 11, myoblasts in all media conditions were passaged, the confluence of the 2.5%, 5%, 7%, and 10% lipid conditions before passage were

90%, 80%, 80%, and 80% respectively. At day 15, a final confluence observation was taken and the growth media was removed and differentiation media was added to induce the formation of myotubes. The final confluence observations for the 2.5%, 5%, 7%, and 10% were 35%, 45%, 50%, and 50% respectively (Fig. 3).

Myotube count

The number of Weddell seal myotubes significantly increased between day 1 and day 7 of differentiation when cultured in media supplemented with 2.5% and 5% lipid mixture (n=6, p=0.027 and p<0.001, respectively). The myotube number in the seal cells differentiated in 5% lipid was observed to have a stepwise increase during the differentiation and was significantly greater at day 7 when compared to day 1 of differentiation (n=6, p<0.001). At day 6 and 7, the cells differentiated in 5% lipid had a significantly greater number of myotubes when compared to the cells differentiated in 7% lipid (n=6, p<0.001). The Weddell seal cells cultured in 7% lipid showed no significant difference in myotube number between day 1 and day 3 (n=6, p=1.000). Between day 4 and 7, there was no significant difference in myotube number in the seal cells differentiated in 7% lipid (n=6, p=0.997). Interesting results were obtained when the number of myotubes was determined for the seal cells cultured in 10% lipid. At day 1 of differentiation the number of myotubes was significantly less than the cells in 2.5%, 5%, and 7% lipid, and remained significantly less (n=6, p<0.001). Starting at day 1, the number of myotubes decreased until day 3 and at day 4 of differentiation in the cells in 10% lipid, the vast majority of myotubes were absent from the cell culture dish, indicating extreme myotube death (Fig. 4).

Myotube width morphology measurements

During day 1 of differentiation (24 hours after growth media was replaced by differentiation media) the Weddell seal myotubes in the 7% lipid group were not significantly different than the myotubes in the 5% lipid condition and were not significantly wider than the myotubes in the 2.5% lipid group (n=6, p=0.076). Day 2 of differentiation, the myotube widths between the different lipid concentrations were not significantly different from each other (n=6, p=0.527). During day 3 of differentiation there was no significant difference in myotube width between each of the three lipid groups (n=9, p=0.852). At day 3 of differentiation, the myotube width in the 2.5% lipid groups did not differ from the widths in day 2 and day 1 (n=6, p=0.107). At day 4 of differentiation, there was no significant difference between the myotube widths between the 2.5%, 5%, and 7% lipid groups (n=6, p=0.510). The myotube width in the 5% lipid condition was not significantly different than day 1-3 (n=6, p=0.882). The myotube width during day 4 in the 2.5% lipid group was not significantly different when compared to day 1, 2, and 3 (n=6, p=0.241). During day 5 of differentiation the myotube widths measured in the 2.5% lipid conditions did not differ from the widths measured during days 2-4 (n=6, p=0.507). In addition, at day 5 of differentiation, there was a significant increase in myotube width between the myotubes in 5% lipid versus 7% lipid (n=6, p=0.008). There was no difference at day 5 between the 2.5% lipid group when compared to the 5% and 7% group (n=6, p=0.231 and p=0.192, respectively). During day 6 of differentiation, we observed similar results as day 5 and the widths were not significantly different from day 5 between all myotubes in the various lipid conditions (n=6, p=0.224). At the last day of differentiation the widths of the myotubes in the 2.5% lipid condition were not different than the widths during day 1-6 (n=6, p=0.278). We did not observe a significant increase in myotube widths at day 7 when comparing the widths

measured in day 1 of differentiation in the 2.5% lipid condition (n=6, p=0.141). At day 7, the myotube width of the 5% lipid group was not significantly different between days 1-3, 5, and 6 (n=6, p=0.105). The myotube width in the 7% lipid condition at day 7 was significantly greater than day 3, 5, and 6 (n=6, p=0.021). The myotube widths of the cells grown in 10% lipid were not measured due to the massive die off of myoblasts before differentiation. Fig. 1.7 displays all myotube width measurements.

Discussion

In the present study we successfully isolated progenitor Weddell seal skeletal muscle cells and through multiple culture experiments, have determined the best media conditions for culturing the myoblasts and differentiating the myoblasts into myotubes. Through this investigation, we found the best cell culture media for Weddell seal skeletal muscle cells is high glucose DMEM growth and differentiation culture media supplemented with a total lipid concentration of 2.5%. To our knowledge, this is the first account of a culture method for primary Weddell seal myoblasts isolated from a muscle biopsy tissue sample.

When growing the Weddell seal myoblasts to be differentiated into functional myotubes, we observed slow growth rates in myoblasts supplemented with higher lipid concentrations (7% and 10%). In contrast, the myoblasts grown in 2.5% and 5% lipid the first passage was as early as day 4 after initial plating. The Weddell seal myoblasts are a primary cell line from mature muscle tissue, which is inherently slow to proliferate because the myoblasts still retain and conform to the natural rules of cell division. This is in direct contrast to immortalized cell lines, which possess faster growth rates. Given the slower than normal proliferation rate of the myoblasts in the 7 and 10% lipid groups as compared the other levels of supplementation it was

concluded that the high lipid content was detrimental to myoblast growth and proliferation (Fig. 1.4). In contrast, the myoblasts growth with media supplemented with 2.5% and 5% lipid gave exceptional proliferation and growth rates, as determined from confluence observations (Fig. 1.5).

The myotubes differentiated in media supplemented with 2.5% lipid resulted in the greatest number of myotubes throughout the differentiation process, and were significantly greater than the other lipid conditions. (Fig. 1.6). The number of seal myotubes differentiated in 7% lipid remained the same throughout the course of the differentiation period, and were lower than the myotubes in 2.5% and 5% lipid (Fig. 1.6). When myotube counts began at day 1, the myotubes differentiated in 10% lipid had the lowest starting values compared to all other lipid supplementation conditions (Fig. 1.6). By day 4, there were no visible myotubes left on the cell culture dish (Fig. 1.4 and Fig. 1.6). Media supplemented with 10% lipid content proved to be extremely toxic to the cells during differentiation. This result is interesting because the cells at the myoblasts stage appeared to grow and proliferate similarly to the myoblasts grown in the 7% lipid condition.

During differentiation of myoblasts into myotubes, myoblasts transform from a skeletal muscle progenitor cell with a single nucleus, into a multinucleated myotube. Myoblast morphology is a triangular shaped cell with a distinct center nucleus, while myotubes appear more tube-like with multiple nuclei situated around the periphery (Minz and Baker, 1967; Scharner and Zammit, 2011). Given that the energy requirements increase as differentiation progresses, we assumed the myotubes would use the high lipid content in the media to fuel myotube development, however, lipid concentrations of 7% and 10% were detrimental to myotube formation and ultimately toxic.

During myotube differentiation, multiple myoblasts merge together to form the functional myotube. The overall width of the resulting myotube is dependent on the number of myoblasts merging together and sharing cell membranes (Allen et al., 1999). With the integration of new myoblasts, the nucleus of the new myoblast is incorporated into the myotube, and takes control over the surrounding cellular functions. The nuclear domain theory suggests myotube width is confined to a narrow range, related to the number of nuclei present in the myotube (Allen et al., 1999). Based on this theory, we conclude the width of Weddell seal myotubes is at the upper limit and myotube width is ultimately based on the number of myoblasts incorporated into the myotube and not the culture media.

Conclusions and Future Directions

In the present study, the best media to culture and differentiate Weddell seal skeletal muscle cells consists of high glucose DMEM supplemented with 2.5% lipid mixture. This concentration of lipid gave us high myoblast proliferation and the greatest total number of myotubes counted. Another important conclusion of the present study was the need to take into account the physiology of the Weddell seal when culturing this unique primary muscle cell line. Weddell seals are highly reliant on lipid oxidation for energy production and the myoblasts also required lipid supplemented to the culture media. 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. Mature myotubes develop with a myogenic program similar to skeletal muscle tissue in whole organisms (Weintraub et al., 1991). Therefore, myotubes exhibit

similar signaling pathways of muscle development including muscle specific proteins such as myoglobin, as well as contractile protein architecture. The present study represents a new method 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. Our research laboratory hopes to use this unique primary Weddell seal skeletal muscle cell line to investigate the molecular regulation of the myoglobin protein. Weddell seals possess high concentrations of this oxygen-binding protein in their skeletal muscle and it is our goal to understand the regulatory mechanisms that drive myoglobin expressions to the great levels seen in this elite diver. In essence, this cell line gives us the ability to conduct experiments on muscle physiology and manipulations we cannot achieve using the whole animal.

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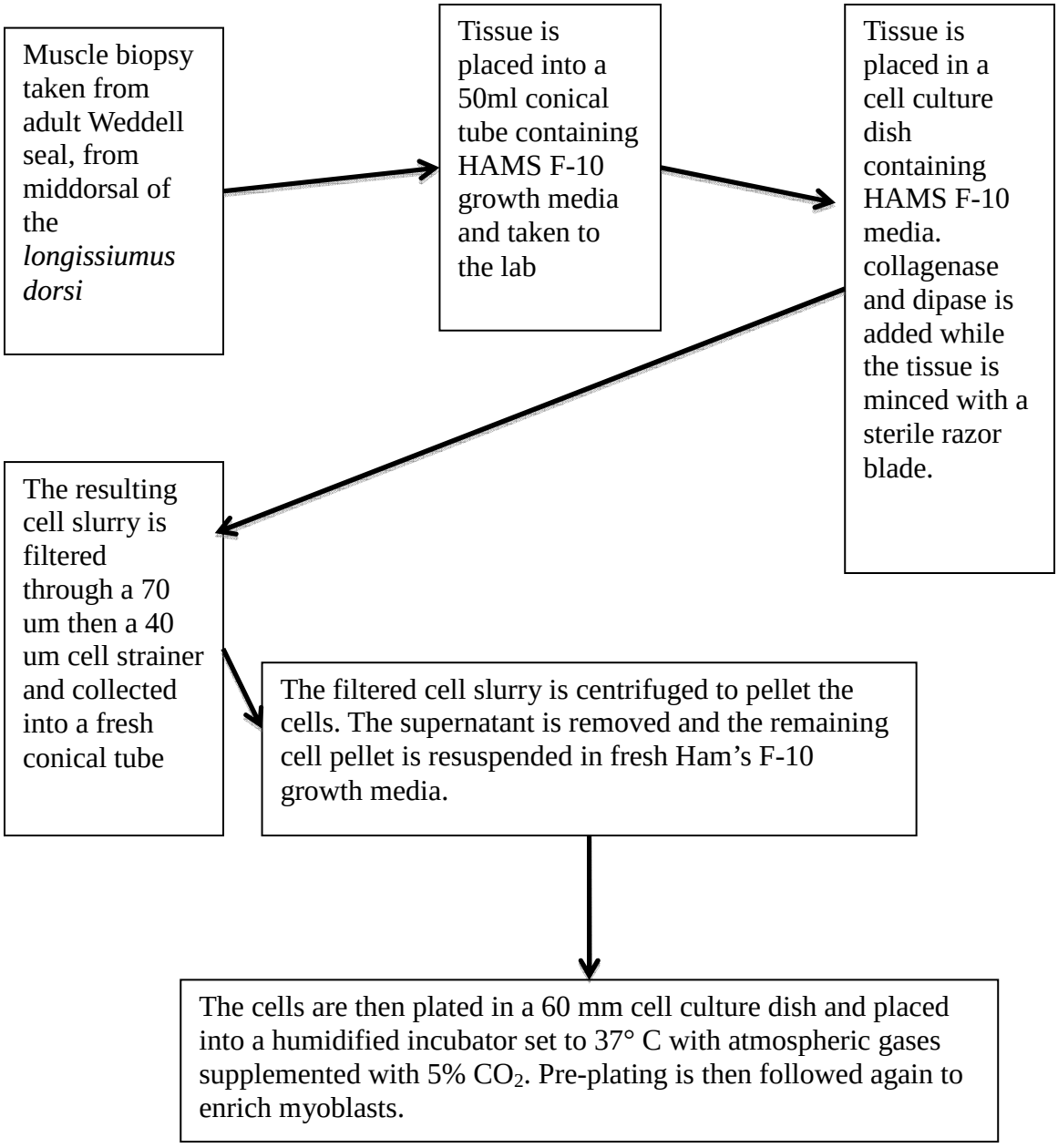

Figure 1.1

Flow chart diagraming the most important steps to the isolation and initial culture of Weddell seal myoblasts from skeletal muscle biopsy tissue.

A

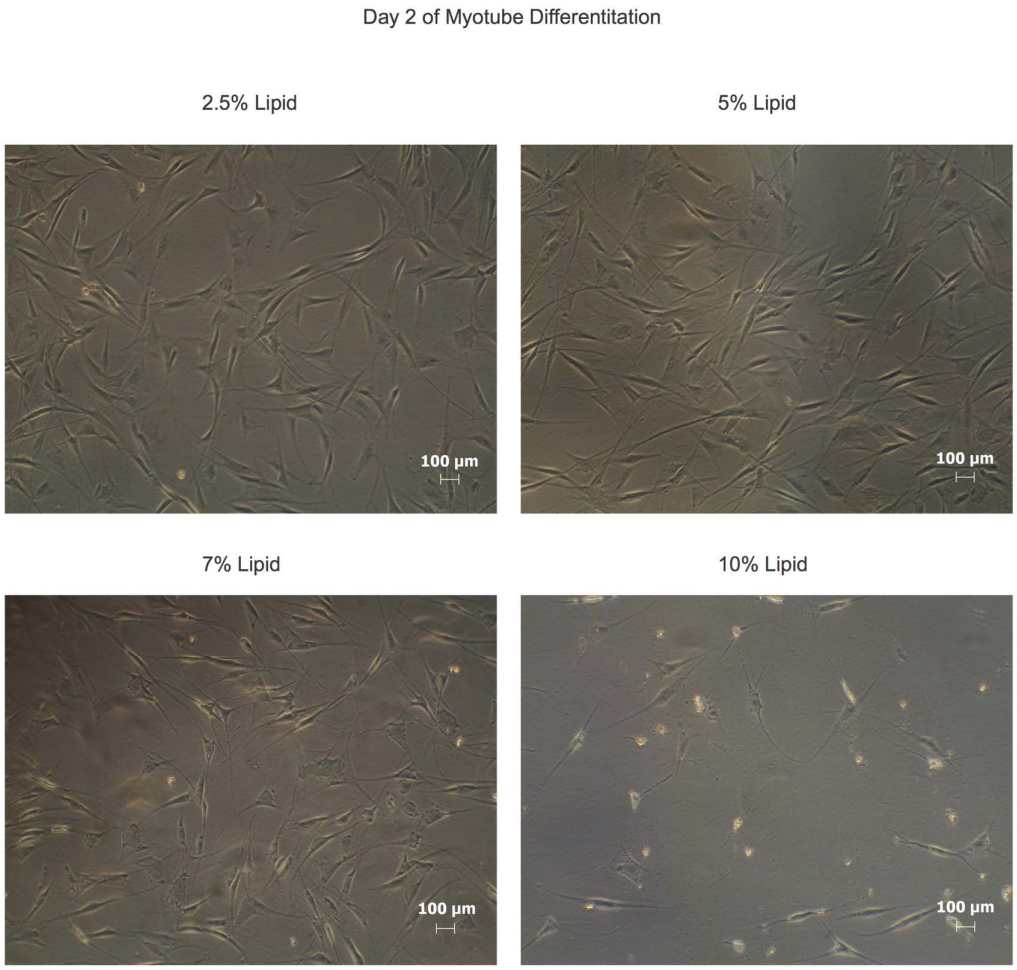

Figure 1.2

(A) 10x magnification of Weddell seal skeletal muscle myotubes after two days of differentiation. Each lipid mixture is represented by one image. Scale bars = 100 µm.

B

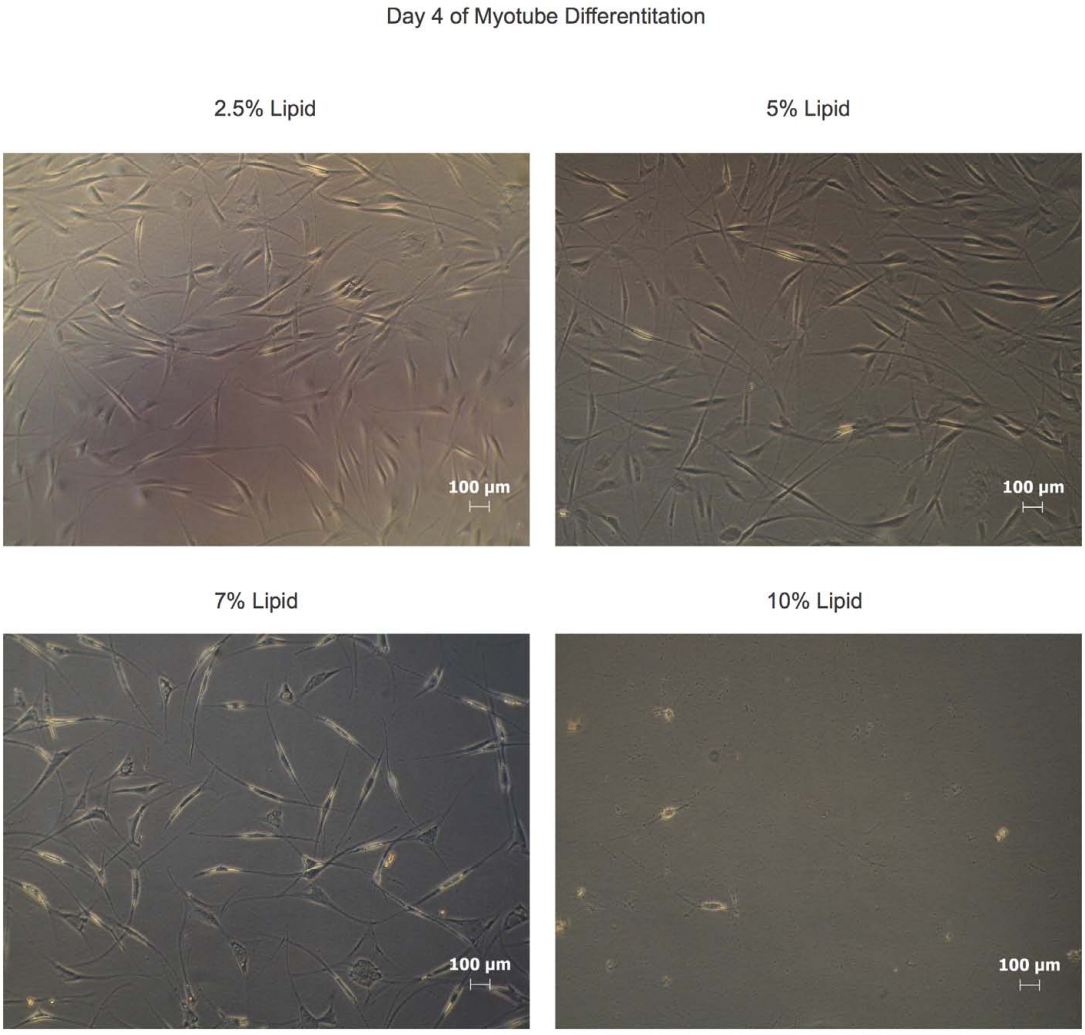

Figure 1.3

(B) 10x magnification of Weddell seal skeletal muscle myotubes after four days of differentiation. . Scale bars = 100 µm.

C

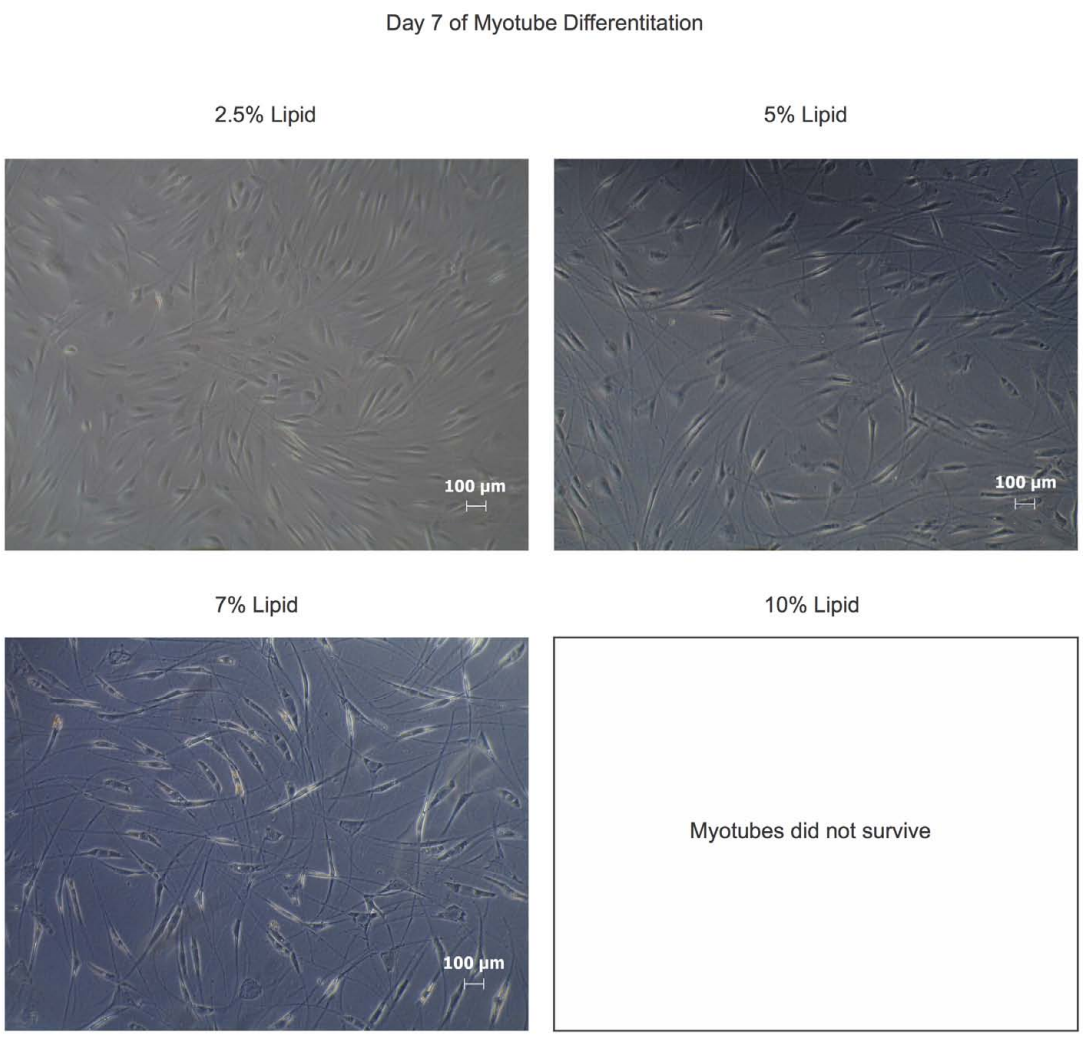

Figure 1.4

(C) 10x magnification of Weddell seal skeletal muscle myotubes after seven days of differentiation. Note the absence of an image for the 10% lipid mixture group as this lipid concentration was lethal to the cells. Scale bars = 100 µm.

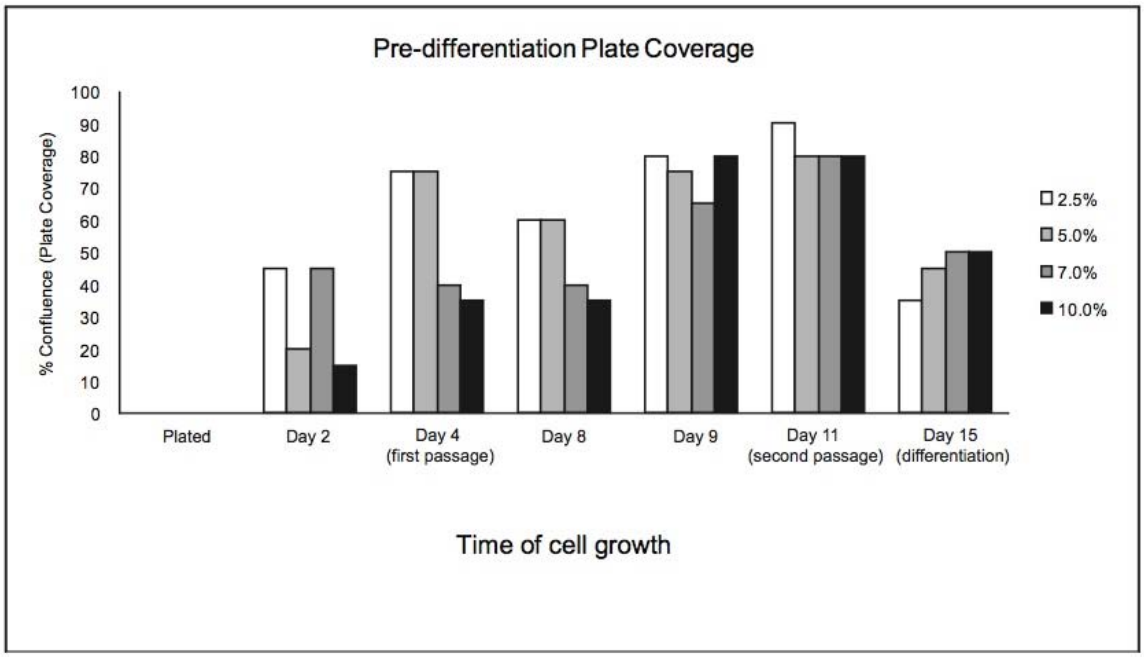

Figure 1.5

Pre-differentiation confluence of the cultured Weddell seal myoblasts starting from day two after initial myoblast plating and ending the day before differentiation. No statistical analysis was performed on plate confluence as the observations were recorded as the percentage of the culture dish coved by myoblasts.

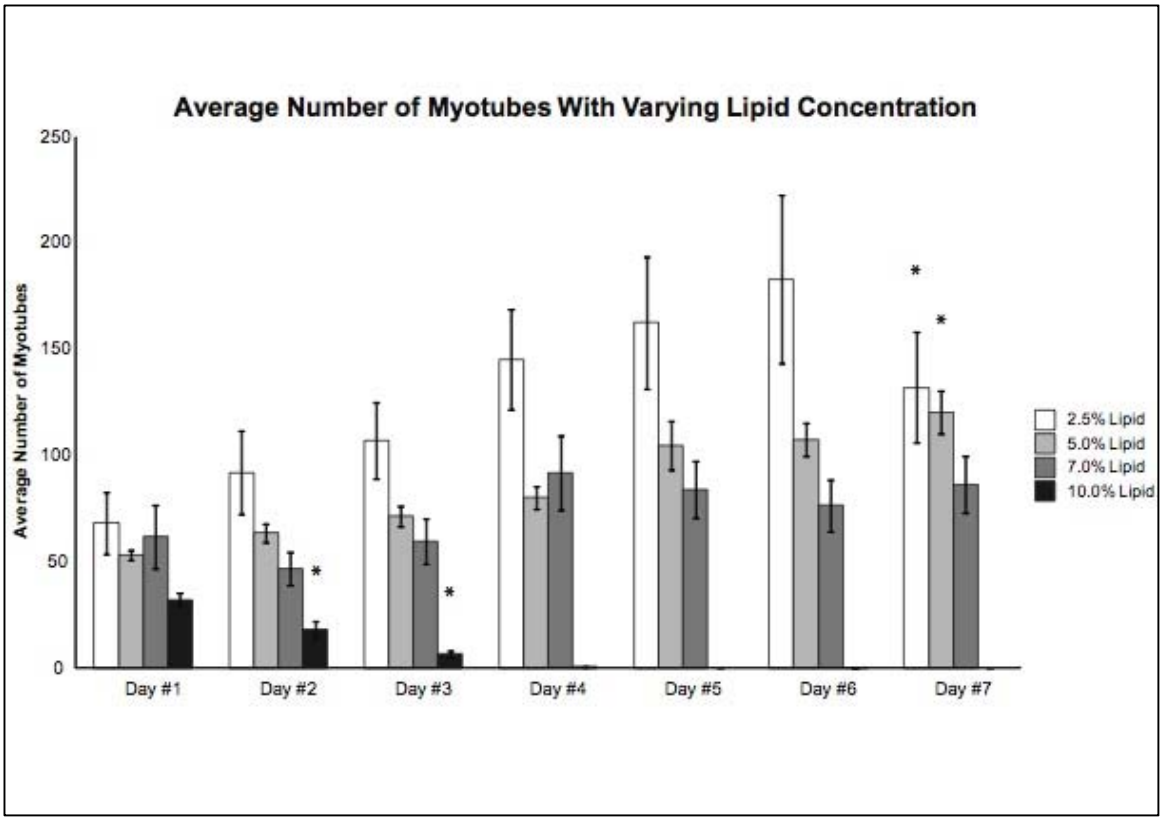

Figure 1.6

Myotube counts from day one of differentiation to day seven. Significant differences between day one and day seven indicated by *. Significance was determined at $p \leq 0.05$, one-way ANOVA repeated measures.

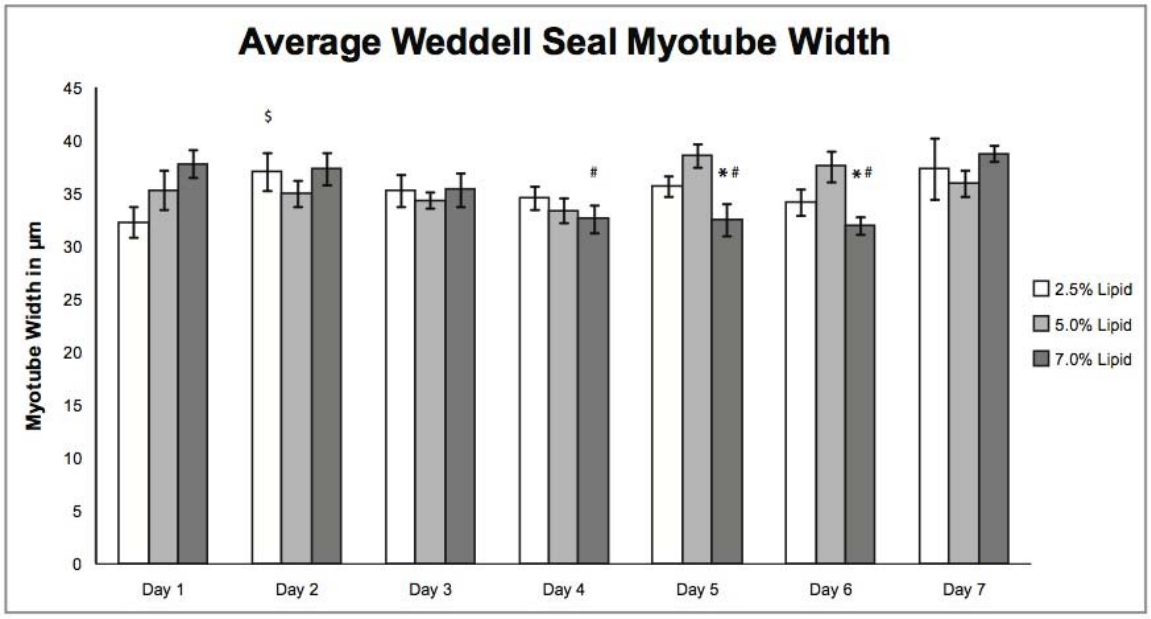

Figure 1.7

Myotube widths of the Weddell seal skeletal muscle cells during the seven days of differentiation. Significant differences between day one and day seven indicated by *. Significant differences between 2.5% and 5% lipid indicated by #. Significance was determined if $p \leq 0.05$, one-way ANOVA.

CHAPTER 3: In the Face of Hypoxia: Myoglobin increases in response to hypoxia and lipid supplementation in cultured Weddell seal skeletal muscle cells

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Summary

A key cellular adaptation to diving in Weddell seals is enhanced myoglobin concentrations in their skeletal muscles, which serve to store oxygen to sustain a lipid-based aerobic metabolism. The aim of this study is to determine if seal muscle cells are inherently adapted to possess the unique skeletal muscle adaptations to diving seen in the whole animal. We hypothesized that the seal skeletal muscle cells will have enhanced concentrations of myoglobin *de novo* that would be greater than those from a C_2C_{12} skeletal muscle cell line and reflect the concentrations of myoglobin observed in previous studies. In addition we hypothesized that the seal cells will respond to environmental hypoxia similarly to the C_2C_{12} cells in that citrate synthase activity and myoglobin will remain the same or decrease under hypoxia and lactate dehydrogenase activity will increase under hypoxia as previously reported. We further hypothesized that β-hydroxyacyl CoA dehydrogenase activity would increase in response to the increasing amounts of lipid supplemented to the culture medium. Our results show that myoglobin significantly increased in response to environmental hypoxia and lipids in the Weddell seal cells, while appearing similar metabolically to the C_2C_{12} cells. The results of this study suggest the regulation of myoglobin expression is fundamentally different in Weddell seal skeletal muscle cells when compared to a terrestrial mammal cell line in that hypoxia and lipids

initially prime the skeletal muscles for enhanced myoglobin expression. However, the cells need a secondary stimulus to further increase myoglobin to levels seen in the whole animal.

Introduction

Myoglobin is a cytoplasmic hemoprotein predominantly found in skeletal and cardiac muscle that is able to reversibly bind oxygen and facilitate the diffusion of oxygen (Garry et al., 2003; Wittenberg and Wittenberg, 2003; Wittenberg et al., 1975). Studies examining the molecular regulation of myoglobin in mouse (*Mus musculus*) skeletal muscle tissue and C_2C_{12} mouse cell culture models have shown myoglobin to be predominantly regulated via the calcineurin/nuclear factor of activated T-cells (NFAT) pathway (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009). Calcium release from the sarcoplasmic reticulum during skeletal muscle activity (contraction) is the essential stimulus to activate the calcineurin enzyme. Activated calcineurin enzyme acts by dephosphorylating NFAT, which translocates into the nucleus to activate target gene programs to promote myoglobin protein expression (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009; Rao et al., 1997). These studies examining myoglobin regulation have demonstrated the importance of skeletal muscle contraction and stimulation in the activation of myoglobin gene expression and regulation in mammalian models.

Myoglobin has been extensively studied in hypoxia-adapted humans and animals to understand its regulation and expression in the skeletal muscle tissue under low oxygen conditions. Historically, it was observed that hypoxia-adapted humans and animals possess elevated concentrations of myoglobin in skeletal and cardiac muscle, presumably because of the

low oxygen environment in which they live (Hoppeler et al., 2003; Renyafarje, 1962). Kanatous and colleagues reported that altered calcium release under environmental hypoxia prevented skeletal muscle myoglobin expression in mouse and mouse cell culture models (Kanatous et al., 2009). Hypoxia as the lone stimulus was shown to cause calcium release from the endoplasmic reticulum, which inhibits the translocation of NFAT into the nucleus, essentially preventing the expression of the myoglobin protein. The study concluded that in the absence of skeletal muscle contraction, myoglobin expression in mouse skeletal muscle decreases or remains the same under hypoxia compared to normoxic oxygen environments. When skeletal muscle is stimulated to contract, selective calcium release from the sarcoplasmic reticulum is able to activate the calcineurin/NFAT pathway, resulting in myoglobin gene expression (Kanatous et al., 2009). When skeletal muscles are exercised under normoxic conditions, myoglobin concentration does not significantly increase beyond pre-exercise values. However, artificially and naturally stimulated skeletal muscles do show a significant increase in myoglobin when coupled with hypoxia (Hoppeler and Vogt, 2001; Kanatous et al., 2009; Mammen et al., 2003; Vogt et al., 2001). These studies, using terrestrial models, have demonstrated that muscle contraction and hypoxia in combination are essential in invoking significant increases in myoglobin expression. Although the terrestrial mouse models featured in the study by Kanatous et al. (Kanatous et al., 2009) showed significant increases in myoglobin under hypoxia, diving mammal models may hold unique mechanisms to further our knowledge regarding myoglobin regulation in response to hypoxia (Kanatous et al., 2009).

Weddell seals, *Leptonychotes weddellii* (Lesson 1826), have developed a suite of unique skeletal muscle adaptations to diving that allow them to maintain muscle function despite increasing ischemia and subsequent increasing tissue hypoxia during long duration breath-hold

dives (Kanatous et al., 2002; Kanatous et al., 2008). An important adaptation Weddell seals possess in order to maintain aerobic metabolism under the hypoxic conditions associated with breath-hold diving is increased concentrations of myoglobin in their skeletal muscles, compared with the skeletal muscles of terrestrial non-diving mammals. Previous studies have measured myoglobin concentrations to be 10-fold greater in the skeletal muscles of diving mammals when compared with those of athletic terrestrial mammals (Hochachka and Foreman, 1993; Kanatous et al., 2002; Reed et al., 1994). High concentrations of myoglobin allow divers to store oxygen within skeletal muscle to fuel aerobic metabolism throughout the duration of the breath-hold dive. Specifically for an adult Weddell seal, myoglobin concentrations of the primary swimming muscle have been calculated to be between 45.9 ± 3.3 and 55.9 ± 2.9 mg myoglobin g^{-1} wet muscle mass (Kanatous et al., 2002; Kanatous et al., 2008; Noren et al., 2005). Weddell seal pups have been found to have less myoglobin than mature adults, yet still possess myoglobin at concentrations of about 35 mg myoglobin g^{-1} wet muscle, which is greater than the concentrations found in the swimming muscle of adult Stellar sea lions (*Eumetopias jubatus*) and Northern fur seals (*Callorhinus ursinus*) (Kanatous et al., 1999; Kanatous et al., 2008). Weddell seal pups are considered to be non-diving during the time they are nursing and relying on their mother for dietary intake. Thus, Weddell seal pups seem to have enhanced myoglobin concentrations without proper skeletal muscle stimulation and exposure to diving conditions, i.e. without the cues normally associated with classical myoglobin regulation pathways. This is also true for other seal species, as studies have found that myoglobin concentrations show a trend of increasing from birth to when the animal is weaned (Burns et al., 2010). While myoglobin expression in terrestrial mouse models appears to be regulated by a combination of several stimuli (hypoxia and skeletal muscle contraction), developing seals already express high levels

of myoglobin before experiencing the same physiological cues. Diving mammals appear to possess an inherent ability to augment myoglobin concentrations to great levels before mature diving patterns develop. Understanding the inherent *de novo* metabolic properties and myoglobin concentrations of developing Weddell seal skeletal muscle cells can advance our understanding of the mechanisms by which these elite divers enhance myoglobin expression to such high levels.

The purpose of this study was to investigate myoglobin concentrations and the metabolic profile of cultured Weddell seal skeletal muscle cells to determine if Weddell seal skeletal muscle cells are inherently adapted to possess enhanced myoglobin concentrations. To accomplish this we examined non-stimulated Weddell seal skeletal muscle cells under normoxic (21% O_2) and hypoxic (0.5% O_2) culture conditions. We measured myoglobin concentrations and the activity of metabolic enzymes in the cells after 7 days of differentiation into myotubes. The enzymes assayed included: citrate synthase (CS), the enzyme in the first step of the citric acid cycle and an indicator of aerobic capacity, lactate dehydrogenase (LDH), the enzyme responsible for the conversion of pyruvate to lactate and an indicator of anaerobic capacity, and β-hydroxyacyl CoA dehydrogenase (HAD), an indicator of β-oxidation of fatty acids. We also examined the role lipids may play in the seal cells to aid in myoglobin expression under each oxygen condition by varying the amounts of lipid (2.5% and 5%) supplemented to the culture medium. The C_2C_{12} mouse muscle cell line served as a control throughout the study and was subjected to the same oxygen conditions as the seal cells. We hypothesized that the Weddell seal skeletal muscle cells would have enhanced concentrations of myoglobin *de novo* that would be significantly higher than those from a C_2C_{12} control mouse cell line and reflect the enhanced concentrations of myoglobin observed in tissue. In addition we hypothesized that the seal cells

would respond to environmental hypoxia similarly to the terrestrial mammalian cell line (C_2C_{12} cells) in that CS enzyme activity and myoglobin will remain the same or decrease under hypoxia and LDH activity will increase under hypoxia as reported in previous studies (Bigard et al., 1991; Kanatous et al., 2009; Lundby et al., 2009; McClelland and Brooks, 2002). We further hypothesized that HAD activity would increase in response to the increasing amounts of lipid supplemented to the growth medium. The results of this study suggest the regulation of myoglobin expression is fundamentally different in Weddell seal skeletal muscle cells when compared to a terrestrial mammal cell line in that hypoxia and lipids may initially prime the skeletal muscle for enhanced myoglobin concentrations; however, the cells are still in need of a secondary stimulus to further increase myoglobin to levels seen in the whole animal.

Materials and Methods

Weddell seal primary skeletal muscle cell isolation

The animal care and use committee at Colorado State University, Fort Collins, CO, USA approved all protocols for this study. MMPA no. 10751788-00. Animal handling and biopsy techniques were performed according to previously published protocols (Kanatous et al., 2008; Trumble et al., 2010). Primary Weddell seal skeletal muscle cells were isolated from the primary swimming muscle (M. longissimus dorsi) of an adult male (8 years old, mass 430 kg) using a muscle biopsy taken on 25 October 2006 near McMurdo Sound, Antarctica. Isolation of the myoblasts was performed in the Albert P. Crary Science and Engineering Center at McMurdo Station. The muscle biopsy tissue was briefly dipped into 70% ethanol and placed into Ham's F-

10 growth media (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and 1% penicillin/streptomycin antibiotic (Sigma Aldrich, St. Louis, MO, USA) and taken *via* snowmobile from the field site on the ice to the Crary Laboratory. The biopsy was placed in a 60 mm cell culture dish and minced with sterile razor blades into 1 mm pieces. The pieces of muscle tissue were placed into a new 60 mm cell culture dish and 3 ml of Ham's F-10 growth medium was added to the plate. The plate was placed into an incubator set to 37° C, 21% O_2, and 5% CO_2. The plate was monitored for myoblast migration for six days. Fresh Ham's F-10 growth medium was added as needed. The cells were then passaged in order to enrich the myoblast cell type. The cells were seeded on a new 60 mm culture dish, after 30 minutes the supernatant containing myoblasts was removed and placed onto a fresh 60 mm culture dish, leaving the fibroblasts behind. After four more pre-plating passages the myoblasts were frozen in liquid nitrogen, and at the end of the field season (November, 2006) taken to Colorado State University, USA. The Weddell seal primary cell line is currently stored in a liquid nitrogen dewar for future use.

Weddell seal skeletal muscle cell culture

The primary skeletal myoblasts isolated from the Weddell seal were grown in standard medium used for skeletal muscle cells containing high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich), 20% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin antibiotic. However, previous studies in our lab found Weddell seals to be highly reliant on fatty acid metabolism (Kanatous et al., 2008). Therefore, the growth and differentiation media were supplemented with Lipid mixture 1 (product number L0288, Sigma

Aldrich). The lipid content of this chemically defined mixture is comprised of the following fatty acids: 2 μg ml^{-1} arachadonic acid and 10 μg ml^{-1} each of linoleic, linolenic, myristic, oleic, palmatic and stearic. The lipid mixture also contained 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. Lipid supplemented to the media at concentrations of 2.5% and 5% produced the best overall cell growth. A lipid concentration of 1% was used initially after myoblast isolation and lipid concentrations increased incrementally until optimum media was achieved. Concentrations that exceeded 5% actually became toxic to the cells and overall myoblast growth rates slowed and eventually ceased. Therefore for this investigation concentrations of 2.5% and 5% were used throughout myoblast growth and differentiation, as this range was greater than the initial concentration yet lower than the lethal limit of the cells. To induce formation of differentiated myotubes, the medium was switched to high glucose DMEM, 5% equine serum, 10 μg ml^{-1} insulin, and 10 μg ml^{-1} transferrin. The differentiation medium contained the same concentrations of lipid supplementation as the growth medium (2.5% and 5%).

Mouse skeletal muscle cell culture

An immortalized C_2C_{12} mouse skeletal muscle cell line was used as a control and was grown and differentiated in parallel with the Weddell seal primary skeletal muscle cells throughout the experiment. The C_2C_{12} myoblasts were grown in high glucose DMEM, 20% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin antibiotic. At 90% confluency, to induce

formation of differentiated myotubes, the medium was switched to high glucose DMEM, 5% equine serum, 10 μg ml^{-1} insulin, and 10 μg ml^{-1} transferrin (Kanatous et al., 2009).

Cell culture oxygen conditions

The cultured skeletal muscle cells were differentiated under normoxic (21% O_2) and hypoxic conditions (0.5% O_2) for 7 days. Cells in the normoxic oxygen condition were kept in a humidified incubator set at 37°C with 5% carbon dioxide. Medium was changed and cells were harvested in a laminar flow cell culture unit (Labconco Corporation Purifier Class II Biosafety Cabinet, Kansas City, MO, USA). Cells in the hypoxic oxygen condition were kept in a humidified hypoxic environmental chamber (Coy Laboratories Products Inc., Grass Lake, MI, USA) set to 37°C with 5% carbon dioxide, 0.5% oxygen, and 94.5% nitrogen. Medium was changed and cells were harvested within the hypoxic chamber, where fluctuations in oxygen concentrations varied by ≤ 0.5% oxygen for periods of 1-2 minutes during the procedure.

Protein harvesting

Muscle cells were harvested using a rubber policeman in lysis buffer containing 79% phosphate-buffered saline, 20% glycerol, 1% Tween-20, 1 mmol l^{-1} dithiothreitol and a protease inhibitor cocktail which included serine and cysteine proteases (Roche Applied Science, Indianapolis, IN, USA). The resulting homogenates were spun at 10,000***g*** at 4°C for 10 min. The supernatant was divided into equal portions and stored at -80°C until assayed. Protein

concentrations were determined using a Pierce Coomassie Plus Protein Assay Reagent (Pierce Chemicals, Rockford, IL, USA).

Enzymatic assays

All enzyme activities were assayed using a BioTek Synergy HT Multi-Detection microplate reader (Winooski, VT, USA). All assay reagents were purchased from Sigma Aldrich.

The assay conditions used were adapted from previous studies (Kanatous et al. 2008 and Reed et al. 1994). CS assay buffers included: 50 mmol l^{-1} imidazole; 0.25 mmol l^{-1} 5,5-dithiobis(2-nitrobenzoic acid) (DTNB); 0.4 mmol l^{-1} acetyl CoA and 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37°C; ΔA_{412}, ε_{412}=13.6. LDH assay buffers included: 50 mmol l^{-1} imidazole; 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C and 1 mmol l^{-1} pyruvate; ΔA_{340}, ε_{340}=6.22. HAD assay buffers included: 50 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} acetoacetyl CoA, and 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; ΔA_{340}, ε_{340}=6.22. CS was assayed to determine the aerobic capacity of the seal and mouse cells. LDH was used to determine the anaerobic capacity of the seal and mouse cells. In addition, LDH activity determined if the seal and mouse cells were experiencing a hypoxic condition (Lundby et al., 2009). HAD activity was assayed to determine the capacity of the seal and mouse cells to oxidize lipids for energy production. Enzyme activity was calculated using the rate of change derived from the maximal linear slope. Activity were normalized to protein concentration and presented as Units mg^{-1} protein.

Myoglobin Concentrations

Myoglobin was assayed using a BioTek Synergy HT Multi-Detection microplate reader following previous methods (Kanatous et al., 1999; Kanatous et al., 2002) (modified from Reynafarje, 1963). In brief aliquots of total protein was diluted in 0.04 mmol l^{-1} potassium phosphate buffer (pH 6.6) and centrifuged for 50 mins at 28,000***g*** at 4°C. The supernatant was removed and bubbled with 99.9% carbon monoxide for 3 min. The absorbance of the supernatant was measured at 568nm and 538nm. Myoglobin concentrations were calculated as previously described (Kanatous et al., 1999) and normalized to total protein concentration. Myoglobin concentrations are presented as mg myoglobin mg^{-1} protein. Horse myoglobin standards were purchased from Sigma Aldrich and measured with each sample.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey *post hoc* test. Significance or alpha was considered at $p \leq 0.05$. All samples were run in triplicate and each enzymatic and myoglobin assay was repeated three times for an overall N=9 for all samples. All statistical tests were performed using SigmaStat version 2.0 (Ashburn, VA, USA). All data are presented as means ± s.e.m.

Results

Cultured Weddell seal cell morphology

Although larger than the C_2C_{12} mouse myoblasts, the cultured Weddell seal myoblasts (Fig. 2.1B) were mononucleated and triangular, and so appeared morphologically similar to control C_2C_{12} mouse myoblasts (Fig. 2.1A). Upon differentiation, the cultured Weddell seal myotubes (Fig. 2.1D) were tube-shaped, which was also similar to the control C_2C_{12} cells (Fig. 2.1C). The transformation of myoblasts into myotubes observed with the Weddell seal cells indicates proper skeletal muscle culture conditions and more importantly, that the Weddell seal primary cell line retained its ability to differentiate into myotubes, because of the presence of the muscle-specific protein myoglobin.

Enzymatic Activities

There was no significant difference observed in CS activity under the normoxic and hypoxic conditions in the Weddell seal cells supplemented with 2.5% lipid (0.066 ± 0.021 and 0.084 ± 0.020 Units mg^{-1} protein, respectively, Fig. 2.2A, $p = 0.554$). Similarly, there was no significant difference in CS activity between the Weddell seal cells supplemented with 5% lipid under the normoxic or hypoxic oxygen conditions (0.100 ± 0.019 and 0.091 ± 0.020 Units mg^{-1} protein, respectively, Fig. 2.2A, $p = 0.758$). In addition, the CS activity of the Weddell seal cells supplemented with 2.5% lipid under the normoxic and hypoxic oxygen condition was not different from that of the Weddell seal cells supplemented with 5% lipid under both oxygen

conditions (Fig. 2.2A). CS activity in the C_2C_{12} control cells was significantly greater under the normoxic and hypoxic conditions when compared with that of the Weddell seal cells in both lipid concentrations (0.182 ± 0.0027 and 0.302 ± 0.0053 Units mg^{-1} protein for normoxia and hypoxia respectively, Table 1, $p < 0.001$).

LDH enzyme activity increased significantly between the normoxic and hypoxic oxygen conditions in the Weddell seal cells supplemented with 2.5% lipid (0.63 ± 0.026 *vs.* 1.66 ± 0.021 Units mg^{-1} protein respectively, Fig. 2.2B, $p < 0.001$) and 5% lipid (0.62 ± 0.012 *vs.* 1.44 ± 0.011 Units mg^{-1} protein respectively, Fig. 2.2B, $p < 0.001$). There was no significant difference in LDH activity between the Weddell seal cells supplemented with 2.5% lipid and 5% lipid under the normoxic condition (0.63 ± 0.026 *vs*. 0.62 ± 0.012 Units mg^{-1} protein respectively, Fig. 2.2B, $p = 0.669$). Under the hypoxic condition, LDH significantly decreased between the Weddell seal cells supplemented with 2.5% lipid and 5% lipid (1.66 ± 0.021 *vs.* 1.44 ± 0.011 Units mg^{-1} protein respectively, Fig. 2.2B, $p < 0.001$). LDH activity in the C_2C_{12} control cells were also significantly greater under the normoxic and hypoxic conditions when compared to the Weddell seal cells in both lipid concentrations (2.769 ± 0.0264 and 3.258 ± 0.058 Units mg^{-1} protein for normoxia and hypoxia respectively, Table 1, $p < 0.001$). LDH activity in the control cells also significantly increased under hypoxia when compared to the normoxic condition (Table 1, $p < 0.001$).

Significant differences were found in HAD activity between the normoxic and hypoxic conditions in the Weddell seal cells supplemented with 2.5% and 5% lipid. In the Weddell seal cells supplemented with 2.5% lipid, HAD significantly increased in the hypoxic condition when compared to the normoxic condition (0.023 ± 0.0002 *vs.* 0.013 ± 0.002 Units mg^{-1} protein respectively, Fig. 2.2C, $p < 0.001$). In contrast, in the Weddell seal cells supplemented with 5%

lipid HAD activity significantly decreased in the hypoxic condition when compared with the normoxic condition (0.036 ± 0.0018 *vs.* 0.041 ± 0.0012 Units mg^{-1} protein respectively, Fig. 2.2C, $p = 0.037$). The Weddell seal cells supplemented with 5% lipid showed significantly increased HAD activity when compared to the Weddell seal cells supplemented with 2.5% lipid under both oxygen conditions (Fig. 2.2C, $p < 0.001$). HAD activity in the control C_2C_{12} cell line was significantly increased under normoxia when compared with that of Weddell seal cells supplemented with 2.5% lipid (Table 1, $p < 0.001$), but was not significantly different from that in seal cells supplemented with 5% lipid (Table 1, $p = 0.540$).

Myoglobin concentrations

Unexpected results were observed when myoglobin was assayed. In the Weddell seal cells supplemented with 2.5% lipid, we observed significantly greater concentrations of myoglobin in the cells under the hypoxic condition as compared to the normoxic condition (0.075 ± 0.0078 *vs.* 0.012 ± 0.0025 mg myoglobin mg^{-1} protein respectively, Fig. 2.2D, $p < 0.001$). When myoglobin concentrations were measured in the Weddell seal cells supplemented with 5% lipid, we observed significantly greater concentrations in the cells under the hypoxic conditions as compared to the cells under the normoxic condition (0.065 ± 0.0068 *vs.* 0.042 ± 0.0067 mg myoglobin mg^{-1} protein respectively, Fig. 2.2D, $p = 0.021$). There was also a significant difference in myoglobin concentration under normoxia between the Weddell seal cells supplemented with 2.5% and 5% lipid (0.012 ± 0.0025 and 0.042 ± 0.0067 mg myoglobin mg^{-1} protein respectively, Fig. 2.2D, $p < 0.001$). This difference was not observed under hypoxia between the different lipid supplementation levels (Fig. 2D, $p = 0.357$). Myoglobin

concentrations in the C_2C_{12} control cells were not significantly different between the normoxic and hypoxic condition (0.031 ± 0.0053 and 0.038 ± 0.001 mg myoglobin mg^{-1} protein respectively, Table 1, $p = 0.366$).

Discussion

The results of this study provide researchers with the first metabolic profile and myoglobin concentrations of cultured primary skeletal muscle cells isolated from a Weddell seal. The main findings from this study are: 1) myoglobin increases under environmental hypoxia in the non-stimulated Weddell seal cells to levels beyond those of the C_2C_{12} control cells, which remained the same under hypoxia; 2) when lipid supplementation was increased from 2.5% to 5% under hypoxia, myoglobin expression also increased in the seal cells; and 3) increasing lipid supplementation from 2.5% to 5% increased myoglobin expression in the seal cells under the normoxic oxygen condition. Contrary to our hypothesis, the cultured myotubes did not reflect the results of other studies using whole muscle tissue in that we did not observe the levels of myoglobin previously reported in past studies (Kanatous et al., 2002; Kanatous et al., 2008; Ponganis et al., 1993). Our results indicate Weddell seal skeletal muscle cells are not inherently adapted to have such high myoglobin concentrations *de novo*; rather, there must be a combination of hypoxia, skeletal muscle activity, and lipids acting in concert to create the unique myoglobin phenotype observed in Weddell seal skeletal muscle.

The most novel result from this study is that un-stimulated Weddell seal cells actually upregulated myoglobin under environmental hypoxia. Under hypoxia, myoglobin concentrations of the Weddell seal cells were found to be up to 1.7 times greater than those of the C_2C_{12} control cells (Table 1). The observed myoglobin concentrations were significantly greater in the Weddell

seal cells (5% lipid) than the C_2C_{12} control cells under both normoxic and hypoxic conditions. This indicates that Weddell seal skeletal muscle cells have a unique response to hypoxia in terms of myoglobin regulation when compared with a terrestrial mammalian cell line. Although it is unknown if the same results would have been observed in other diving marine species' skeletal muscle cells as a result of the uniqueness of the primary cell line used in this study, the result shows a clear capacity of Weddell seal muscle cells to respond to hypoxia in an adaptive fashion by upregulating myoglobin. The principle finding of this study, that Weddell seal cells upregulate myoglobin under hypoxia, is in direct contrast to recent studies undertaken to understand myoglobin expression in mouse models and mouse cell culture (Kanatous et al., 2009).

It has been shown using C_2C_{12} cells and whole animal mouse models, that chronic environmental hypoxia as the lone stimulus is not sufficient to increase myoglobin expression (Kanatous et al., 2009). It was only when the skeletal muscle was stimulated to contract under hypoxia that an increase in myoglobin was measured, indicating the need for contraction to activate myoglobin gene expression. The key factor in the expression of myoglobin under hypoxia is the need for simultaneous stimulation of specific calcium handling pathways associated with skeletal muscle activity (Kanatous et al., 2009). This is consistent with other studies that measured an increase in myoglobin mRNA content after providing skeletal muscle stimulation under simulated hypoxia (Hoppeler and Vogt, 2001; Vogt et al., 2001). Under normoxia however, skeletal muscle activity (exercise) was not sufficient to increase myoglobin concentrations in exercising human subjects and trained laboratory rats. These studies show myoglobin expression was unaffected by the demands of the exercising subject under normoxia, which may indicate the need for an additional stimulus to enhance myoglobin concentrations

(Harms and Hickson, 1983; Masuda et al., 1999). It is important to note that changes in myoglobin have been shown to occur with and without activity-induced changes in skeletal muscle fiber type. In some cases myoglobin has been shown to increase in response to changes in fiber type during simulated exercise under normoxic conditions (Chin et al., 1998). In contrast, Kanatous and colleagues showed decreases in myoglobin under hypoxia without simultaneous changes in fiber type (Kanatous et al., 2009); however, it is unknown whether any changes in fiber type occurred in the Weddell seal skeletal muscle cells with the changes in myoglobin.

The relationship between skeletal muscle activity and hypoxia and its role in myoglobin expression has been explored in mammalian and non-mammalian breath-hold divers. Studies following the ontogeny of diving in mammalian species have shown that the highly active juvenile Weddell seals have significantly greater myoglobin concentrations than adults, which is presumed to be due to the high skeletal muscle activity during multiple breath-hold dives (Burns, 1999; Kanatous et al., 2008; Kanatous et al., 2009). In non-mammalian models using emperor penguins (*Aptenodytes forsteri*) it was shown that captive penguins, which have not experienced long breath-hold dives, had lower myoglobin concentrations in their primary swimming muscle than penguins from the wild (Ponganis et al., 2010). In addition, myoglobin mRNA content was found to be lower in pre-fledging non-diving chicks when compared to diving adults (Ponganis et al., 2010). These results from mammalian and non-mammalian divers indicate the need for a hypoxic dive bout, coupled with the skeletal muscle activity associated with swimming to enhance myoglobin expression.

Another result of significant interest from this study is that the addition of lipid to the growth and differentiation media of the Weddell seal cells enhanced myoglobin concentrations under normoxic oxygen conditions. Under normoxia, a 1.3 times increase in myoglobin

expression in the Weddell seal cells was measured as compared to the C_2C_{12} control cells was measured when the medium was supplemented with lipid at a concentration of 5%. Our results show that lipids may prime skeletal muscle initially to allow Weddell seals to enhance myoglobin concentrations to levels well beyond those of terrestrial mammals. This finding may explain why non-diving weaned Weddell seal pups have myoglobin concentrations up to 35 mg g^{-1} wet tissue in their primary swimming muscle (Kanatous et al., 2008). Weaned Harp seal (*Pagophilus groenlandicus*) and Hooded seal (*Cystophora cristata*) pups have ~30% of the myoglobin content of adults in their skeletal muscle while weaned Weddell seal pups specifically have 35.5 ± 3 mg myoglobin g^{-1} wet tissue or ~63% of that of adults (Burns et al., 2010; Kanatous et al., 2008). During development, seal pups rely on milk with a high fat content (>50%) and relatively low carbohydrate content as an energy source before commencement of their first breath-hold dive (Burns et al., 2010; Oftedal, 1993). During this pre-dive development, seal pups show increasing myoglobin expression before becoming expert adult divers, indicating dietary lipids may be the key initial stimulus to possessing enhanced myoglobin stores (Burns et al., 2010; Kanatous et al, 2008; Lestyk et al., 2009; Noren et al., 2005). This idea is illustrated in developing harp seal pups when myoglobin was analyzed during fetal to late weaned time points. Harp seals are born relatively altricial when compared to other pack-ice seals and spend about nine days on land nursing before the commencement of diving (Ronald and Dougan, 1982). During this time, when high fat milk was the only source of dietary intake, myoglobin was found to significantly increase from the fetal stage to a late weaned stage (Burns et al., 2010). The results from the present study taken with the results from harp seals (Burns et al., 2010) demonstrate the fundamental role lipids may play during the development of enhanced myoglobin stores in skeletal muscle of marine mammals.

A fundamental role for lipids in the development of myoglobin may also translate to non-mammalian breath hold divers. Emperor penguin chicks (3-6 months old) have been shown to possess relatively high myoglobin content before breath-hold diving. Because of the lack of skeletal muscle activity and hypoxia associated with diving, lipids from the diet of penguin chicks may be the key stimulus to augment myoglobin content up to 2.7 ± 0.4 g 100 g^{-1} muscle in 6 month old chicks (Ponganis et al., 2010). In pre-fledging emperor penguins, the occasional wing flapping to aid in maintaining body temperature coupled with dietary lipid, may be responsible for the increases in myoglobin as the penguin develops into the diving adult stages of its life (Ponganis et al., 2010). Results from Ponganis and colleagues (Ponganis et al., 2010) are similar to those of this study because we found myoglobin to significantly increased in the seal cells under the normoxic oxygen condition, when we increased lipid concentrations from 2.5% to 5% (Fig. 2.1D). Our results suggest a complex interaction between myoglobin and lipids is present in divers and lipids may enhance myoglobin expression initially during the development of breath-hold divers. However, we hypothesize that there is still the need for additional external cues (hypoxia and skeletal muscle activity) to further stimulate the increase in myoglobin expression to levels seen in mature animals.

The enzyme activities showed classical responses to environmental hypoxia, except when we measured CS activity in the cells supplemented with 2.5% lipid. We measured a trend of increase in CS activity under hypoxia but this was not statistically significant. The increase actually correlated with an increase in HAD activity, suggesting an increase in flux of acetyl-CoA from lipid oxidation through the metabolic system. When we measured LDH, we observed a significant upregulation of LDH under hypoxia in the Weddell seal cells with 2.5 and 5 % lipid, suggesting an increase in anaerobic capacity. We measured the same significant increase in

LDH activity under hypoxia in the C_2C_{12} control cell line (Table 1). Previous studies measuring LDH activity in skeletal muscle of hypoxia-adapted mammals have also shown an increase in activity of the enzyme (Bigard et al., 1991; Lundby et al., 2009; McClelland and Brooks, 2002). This result is significant in that we observed evidence that the cells are actually experiencing a hypoxic situation, in which the response was to increase anaerobic capacity. More importantly, the LDH results show the Weddell seal cells and C_2C_{12} mouse cells are similar in their anaerobic response to environmental hypoxia, yet dissimilar in terms of the myoglobin expression response to hypoxia. When we compared CS and LDH activities of our Weddell seal cells (2.5% and 5% lipid supplementation) to our control C_2C_{12} cell line, we observed that the activity of both enzymes was significantly lower under the normoxic and hypoxic conditions (Table 1). The overall superior metabolic profile of the C_2C_{12} cell line was attributed to the properties of an immortalized cell line with optimized growth and differentiation media, which give the cells the best chance to grow into well-developed myotubes (Blau et al., 1983). Although the C_2C_{12} cells appeared to be in better position to express more myoglobin by having a greater capacity to generate usable energy through aerobic metabolism, they actually had significantly less myoglobin than the Weddell seal cells.

In this study, environmental hypoxia and the addition of lipids to cell culture media, was sufficient to increase myoglobin in non-stimulated cultured Weddell seal skeletal muscle cells. The results suggest fundamental differences at the cellular level in myoglobin regulation in Weddell seal skeletal muscle cells when compared to skeletal muscle cells of a terrestrial mammal. Although the cells enhanced myoglobin expression to levels greater than those in a control C_2C_{12} mouse cell line (Table 1), we did not measure the amounts seen in previous studies of Weddell seals. The results of the present study also showed that Weddell seal skeletal muscle

cells increase myoglobin protein expression in response to increasing amounts of lipid added to the growth and differentiation media. These results suggest myoglobin regulation in cultured Weddell seal cells may initially be independent of skeletal muscle activity and rely on lipids to prime the skeletal muscle. However, to enhance myoglobin protein expression to levels seen in the whole animal, a secondary stimulus of hypoxia coupled with skeletal muscle activity is still needed. This study, which utilized a cell culture technique on a marine mammal, has revealed novel data that advance our understanding of enhanced myoglobin expression of elite diving mammals.

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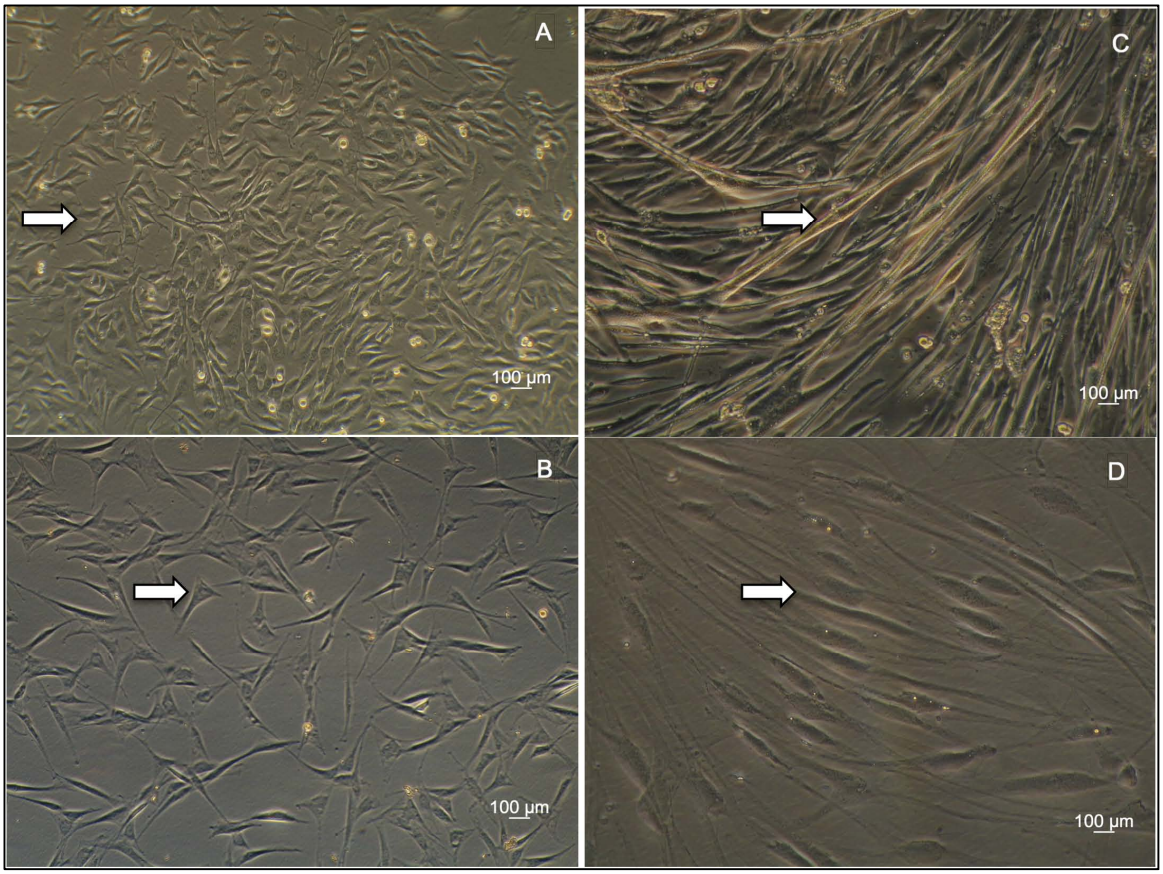

Figure 2.1

A) Control mouse skeletal muscle cell line (C2C12) myoblasts. (B) Primary Weddell seal skeletal muscle cell myoblasts. (C) Control mouse skeletal muscle cell line (C2C12) fully differentiated into myotubes. (D) Primary Weddell seal skeletal muscle cells fully differentiated into myotubes. Magnification for A–D, 20x. Arrows indicate individual myoblasts and myotubes. Scale bars, 100 µm.

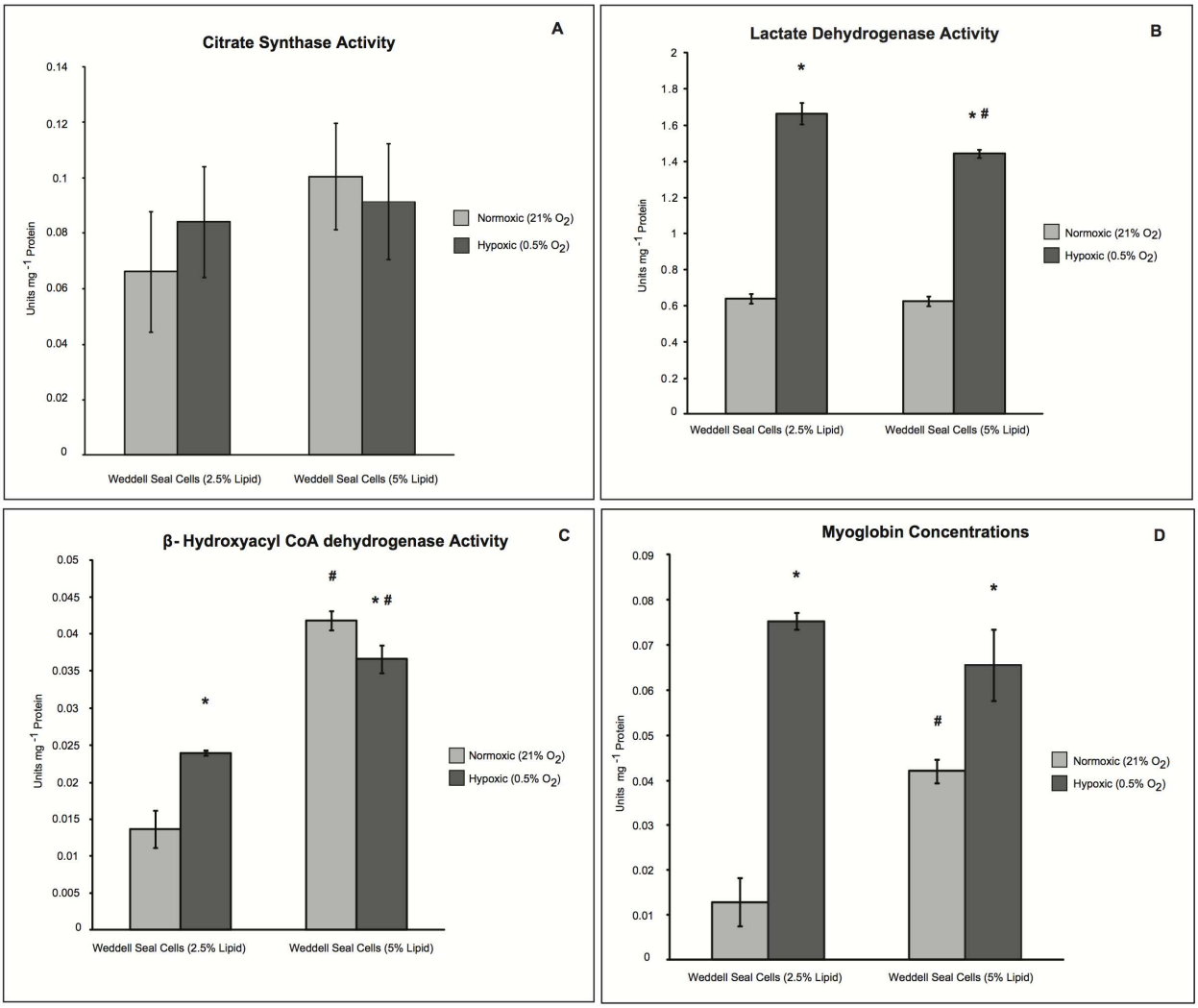

Figure 2.2 Enzymatic activities and myoglobin concentrations in the cultured Weddell seal skeletal muscle cells under normoxic (21% O_2) and hypoxic (0.5% O_2) conditions and 2.5% and 5% lipid supplementation. Citrate synthase (A), lactate dehydrogenase (B), and β-hydroxyacyl CoA dehydrogenase (C) activity, and myoglobin concentration (D) are presented as means ± s.e.m. Significant difference between the normoxic and hypoxic conditions is indicated by *. Significant difference between the Weddell seal cells grown in 2.5% lipid and 5% lipid is indicated by #. N = 9 for all enzymatic and myoglobin assays.

Table 1. Summary of enzymatic activities and myoglobin concentrations of Weddell seal skeletal muscle cells and control mouse skeletal muscle cells (C_2C_{12}). Enzyme activities are presented as Units mg^{-1} protein. Myoglobin concentrations are presented as mg myoglobin mg^{-1} protein. All values are presented as means ± s.e.m. Significant difference from the normoxic (21% O_2) oxygen condition indicated by *. Significant difference between the Weddell seal cells grown in 2.5% lipid and 5% lipid indicated by #. Significant difference between C_2C_{12} control cell line and Weddell seal cells indicated by $.

		Weddell seal		C_2C_{12}
		2.5% lipid	5% lipid	
Normoxic (21% O_2)	CS	0.066 ± 0.021	0.100 ± 0.019$^{\#}$	0.182 ± 0.0027$^{\$}$
	LDH	0.63 ± 0.026	0.62 ± 0.012	2.769 ± 0.0264$^{\$}$
	HAD	0.013 ± 0.002	0.041 ± 0.0012$^{\#}$	0.030 ± 0.0017$^{\$}$
	Myoglobin	0.012 ± 0.0025	0.042 ± 0.0067$^{\#}$	0.031 ± 0.0053$^{\$}$
Hypoxic (0.5% O_2)	CS	0.084 ± 0.022	0.091 ± 0.020	0.302 ± 0.0084$^{\$*}$
	LDH	1.66 ± 0.021*	1.44 ± 0.011$^{*\#}$	3.258 ± 0.058$^{\$*}$
	HAD	0.023 ± 0.0002*	0.036 ± 0.0018$^{*\#}$	0.050 ± 0.0050$^{\$*}$
	Myoglobin	0.075 ± 0.0078*	0.065 ± 0.0068*	0.038 ± 0.001$^{\$}$

Data presented at means ± s.e.m. Enzyme activities are presented as Units mg^{-1} protein. Myoglobin concentrations presented as mg myoglobin mg^{-1} protein.

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CHAPTER 4: Conclusion

The results of these studies represent the first attempt to determine myoglobin expression in Weddell seals using cultured primary skeletal muscle cells. The results provide researchers with an optimized protocol for the isolation and subsequent culture of Weddell seal skeletal muscle cells as well as the first metabolic profile and myoglobin concentration measurements of the differentiated Weddell seal myotubes. The main findings of this thesis include: 1) To optimize the growth and differentiation conditions for the cell line, the culture media requires lipid supplementation at concentrations of 2.5% and lipid concentrations greater than 7% were toxic to the cells. 2) Myoglobin concentration increases in the Weddell seal cells under hypoxia to levels greater than C_2C_{12} control cells under the same hypoxic condition. 3) Under hypoxia when lipid was increased from 2.5% to 5% in the Weddell seal cells, myoglobin expression also increased. 4) Increasing lipid supplementation from 2.5% to 5% also increased myoglobin expression under normoxic conditions in the seal cells.

Contrary to the original hypotheses, the cultured seal skeletal muscle cells required lipid supplemented in the growth and differentiation media; and myoglobin concentrations of the cultured seal cells did not support previous studies in that the cells did not possess enhanced concentrations of myoglobin previously reported in whole animals (Kanatous et al., 2002; Kanatous et al., 2008). Interestingly, seal cell culture requires lipid to be supplemented to the growth media in order to create the optimum culture conditions. This indicates the need to represent the animal's unique cell physiology when attempting to culture a primary cell line from a unique model organism, such as the Weddell seal. Surprisingly, the results indicate that Weddell seal skeletal muscle cells are not inherently adapted to possess high myoglobin

concentrations *de novo*, rather there must be a combination of hypoxia, skeletal muscle activity, and lipids acting in concert to create the enhanced myoglobin concentrations observed in Weddell seal skeletal muscle.

When culturing the unique primary cell line, growth media traditionally used for a C_2C_{12} cell line was first tested. This media consists of mostly high glucose DMEM that provides glucose and amino acids that are used for cellular growth. A high glucose media recipe is essential for glucose uptake by C_2C_{12} cells with maximal rates reaching 4200 pmol (min mg protein)$^{-1}$ (Sarabia et al., 1990). However, traditional mouse cell culture media does not contain fatty acids, which are essential to the metabolism of Weddell seals. Based on the citrate synthase: β-hydroxylacetoacyl CoA enzyme ratio, which is commonly used as an index of the overall contribution of lipid oxidation to total aerobic metabolism, Weddell seals are almost completely reliant on lipid oxidation for energy production, with a ratio in the primary swimming muscle at 0.3 (Kanatous et al., 2002). The study presented in chapter two, illustrates the need for the addition of lipids to the seal cell culture media to obtain the optimum growth potential for the muscle cells, as the major contributor of energy production is from fatty acid oxidation. The major conclusion from this specific experiment was the need to take into account the whole animal physiology when creating optimum growth media for use in unique nontraditional culture systems. For the Weddell seal, the results indicate growth and differentiation media containing high glucose DMEM supplemented with fatty acids at a final concentration 2.5% of the total media volume was the optimum recipe to achieve the fastest growth rates of the myoblasts, and the largest myotubes.

Lipid concentrations above 5% of the media resulted in slow myoblast proliferation and a reduced number of differentiated myotubes. This result was illustrated by the cytotoxic effects a

lipid concentration of 10% had on Weddell seal myotube formation. It is interesting to note that during myoblast proliferation, the 10% lipid condition was not toxic to developing myoblasts; rather it was toxic upon differentiation into mature myotubes. This result suggests that Weddell seal myoblasts are able to tolerate high lipid concentrations, but when differentiated into myotubes, the high lipid content of the media proves to be lethal to the cell.

An interesting result of the experiment presented in chapter three is that the cultured Weddell seal skeletal muscle cells increased myoglobin expression under hypoxia to levels 1.7 fold greater than those of the control C_2C_{12} cell line. This result indicates there must be differences in the adaptive regulation of myoglobin protein expression under hypoxia between the Weddell seal cell line and a terrestrial mouse cell line. The results of Kanatous and colleagues (Kanatous et al., 2009) show a down-regulation of myoglobin in un-stimulated mouse cell culture models (C_2C_{12}) when exposed to environmental hypoxia, indicating an independent interaction between hypoxia inducible factor (HIF) and myoglobin expression, which is further supported by the absence of a HIF binding site on the myoglobin promoter. HIF is a transcription factor that is stabilized under low oxygen conditions and activates genes associated with enhanced oxygen delivery (Ratcliffe et al., 1998; Wenger, 2002). The adaptive responses to HIF expression under hypoxia in rodent species, include increased capillary density, increased hematocrit volume, and reduced muscle fiber diameter (Kanatous et al., 2009). The main goal of these adaptive responses to hypoxia is to increase oxygen delivery to working skeletal muscle. Interestingly, myoglobin, which is an oxygen binding protein in skeletal and cardiac tissue is unaffected by the HIF transcription factor, as the myoglobin gene appears to lack a HIF binding site. Studies investigating the regulatory binding sites on the myoglobin gene promoter region have implicated Sp1, MEF2, and NFAT proteins as the main transcription factors essential for

myoglobin gene expression (Chin et al., 1998; Garry et al., 2003; Grayson et al., 1995; Yan et al., 2001). Given the myoglobin gene does not appear to contain a HIF binding site on the promoter, it is very interesting that the differentiated Weddell seal cells were able to increase myoglobin expression to levels beyond the control cells under environmental hypoxia. This result suggests a fundamental difference in the molecular regulation of myoglobin in Weddell seal myotubes when compared to myotubes of a terrestrial mammal origin when a hypoxic situation is presented.

Another interesting result from this study is lipid supplemented to the growth and differentiation media enhanced the expression of myoglobin in the Weddell seal cells under normoxic oxygen conditions. When lipid supplementation was increased to 5% of the total media, a 1.3 fold increase in myoglobin expression was measured as compared to the C_2C_{12} control cells. Lipids may have a significant role in developing enhanced myoglobin stores in young marine mammals. Weddell seal pups rely completely on their mother's milk as a source of dietary intake as they are not foraging or diving during this pre-dive development. Seal milk has a very high fat content, which has been measured to be >50% in some species (Burns et al., 2010; Oftedal, 1993). Weddell seal pups have myoglobin contents of 35 mg g^{-1} wet tissue in their skeletal muscles, which is about 65% of that of adults (Burns et al., 2010; Kanatous et al., 2008). Essentially, they are not experiencing the skeletal muscle activity associated with diving, so they are lacking the classic cues associated with myoglobin regulation (activated calcineurin/NFAT pathway), yet still have very high concentrations of myoglobin in the skeletal muscle. It appears that Weddell seal pups possess an inherent ability to augment skeletal muscle myoglobin when they are not preforming hypoxic dive bouts and while they are relying only on high fat milk for energy. This indicates lipids from dietary milk may be an essential stimulus to prime seal pups to

have enhanced myoglobin stores during the initial pre-diving life stage. The results of chapter three reflect this hypothesis by showing a significant increase in myoglobin concentration in the Weddell seal myotubes when the amount of lipid was increased from 2.5% to 5%. This increase was measured in the normoxic condition, indicating myoglobin increases in Weddell seal myotubes absent of a hypoxic situation when lipid supplementation increases. A lipid concentration of 5% was sufficient to increase myoglobin concentration to levels significantly greater than the C_2C_{12} control cell line.

The enzyme activities measured in both the Weddell seal and C_2C_{12} myotubes showed classic responses to environmental hypoxia as indicated by increasing lactate dehydrogenase activity. In addition, β-hydroxyacyl CoA dehydrogenase, an indicator of lipid oxidation increased when increasing amounts of lipid were added to the culture media. Overall enzymatic activities in the Weddell seal myotubes were lower than those of the C_2C_{12} control cell line. The superior metabolic capacity of the control myotubes was attributed to the properties of an immortalized cell line (Blau et al., 1983). However, it is interesting to note that the control cell line appeared to possess the ability to express more myoglobin, by having the ability to produce more energy via aerobic metabolism, though they actually had less myoglobin than the Weddell seal cells.

Perspectives

This thesis concludes that environmental hypoxia and the addition of a lipid supplementation to cell culture media, working in concert, was sufficient to increase myoglobin protein expression in cultured non-stimulated Weddell seal skeletal muscle cells. These findings

are in direct contrast with previous studies exploring myoglobin regulation in terrestrial mouse models and immortalized terrestrial mouse cell lines. The results suggest fundamental differences in the regulation of myoglobin of Weddell seals at the developing myoblast and differentiated myotube phases. The regulatory mechanisms of myoglobin in cultured Weddell seal skeletal muscle cells may initially be independent of skeletal muscle activity and rely on lipids to prime enhanced myoglobin expression. However, it is hypothesized that there must be a unique combination of hypoxia, lipids, and skeletal muscle activity working together to drive myoglobin concentrations to great levels reflecting what is observed in the skeletal muscle tissue of mature Weddell seals. As shown in this thesis, to culture skeletal muscle cells of unique organisms to study specific adaptations, the organism's physiology must be taken into account when devising a culture method. When tailoring culture protocols, creating a media that can match the organism's energy requirements is essential to cell survival. By creating specific culture methods, researches can presumably create cell lines from a multitude of organisms to perform *in vitro* experiments to investigate unique adaptations. It is hoped that the information gleaned from these studies can further current knowledge of marine mammal adaptations to breath-hold diving and influence other researchers to include new techniques to investigate the regulatory pathways involved in enhanced myoglobin expression.

Future Directions

The results presented in the current thesis advance our understanding of *de novo* myoglobin expression in elite breath-hold divers and shows great success using a cell culture technique to investigate the intricate molecular regulatory factors. The next steps to elucidate the

regulatory factors include analysis using advanced proteomic techniques such as 2D gel electrophoresis. Using this technique on isolated nuclear proteins, it can be hypothesized that differences in regulatory or transcription factors, specifically calcineurin and NFAT, may be found between Weddell seal skeletal muscle cells and C_2C_{12} cells, which may explain the differences in myoglobin concentrations observed. To investigate the hypothesis of hypoxia, lipids, and skeletal muscle activity working together to enhance myoglobin expression, an experiment using stimulated Weddell seal cells under hypoxia and various amounts of lipids can be performed to determine changes between a stimulated and non-stimulated group. The stimulation of myotubes in culture can be achieved by the addition of calcium or caffeine to the culture media at timed intervals to activate calcium release from the sarcoplasmic reticulum. To further investigate the relationship between lipids and myoglobin expression, separate tests with varying types of lipids such as, monounsaturated and polyunsaturated fatty acids may be used to supplement the culture media, testing the hypothesis that the increase in myoglobin expression are due to a specific type of lipid. The future experiments that can be performed are based on the results of this thesis and continue to use *in vitro* methods to determine the intricate molecular pathways that regulate enhanced myoglobin protein expression in Weddell seals.

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