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Consideration of Diet Was Necessary for the Successful Isolation and Culture of Weddell Seal (*Leptonychotes weddellii*) Myoblasts

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Abstract

We developed an isolation and culture method for Weddell seal (*Leptonychotes weddellii*) myoblasts and discovered that isolated Weddell seal cells proliferate poorly after initial plating and freezing without lipid supplementation in media, which is reflective of their high-fat diets. We tested the effects of media lipid concentrations ranging from 2.5 to 10% on the number of myotubes formed between days 1 and 7 of differentiation. We determined that the growth and differentiation media for cultured Weddell seal skeletal muscle cells must be supplemented with a lipid concentration between 2.5 and 5%. This modification to standard growth and differentiation media formulations illustrates the importance of considering the diet of non-model organisms in primary culture. The success of these techniques provides a unique opportunity to investigate molecular regulatory pathways essential to physiological responses seen in the whole animal.

Key Words: primary cell culture, Weddell seal, *Leptonychotes weddellii*, myotubes, diet, lipid

Introduction

The Weddell seal (*Leptonychotes weddellii*) utilizes aerobic metabolic pathways during breath-hold exercise while diving (Kooyman et al., 1980; Williams et al., 2000). Weddell seals and other pinnipeds have high myoglobin concentrations in their swimming muscles, ranging from 22.4 mg/g in a northern fur seal to 55.9 mg/g in the adult Weddell seal; for comparison, a cotton rat has 1.7 mg/g (Kanatous et al., 1999, 2008). This allows for a large oxygen store to draw from during dives, facilitating the use of lipid-based aerobic pathways while decreasing anaerobic respiration and limiting lactate production (Kanatous et al., 2002). To further study the development of myoglobin regulation in a diving mammal, our lab developed a protocol to isolate and culture Weddell seal primary skeletal muscle cells and determined media conditions for growth and differentiation by considering the Weddell seals' diet.

The diet of the Weddell seal depends on age; varies spatially and temporally; and can include pelagic and benthic fish, cephalopods, and crustaceans (Plotz, 1986; Burns et al., 1998; Lake et al., 2003). Lenky et al. (2012) examined the proximate composition of several species of notothenniid and myctophid fishes known to be Weddell seal prey. The pelagic *Pleuragramma antarcticum,* one of the Weddell seals' predominant prey species in the Weddell Sea, McMurdo Sound, Mawson Coast, the northern Vestfold Hills, and the Danco Coast of the Antarctic Penninsula, was found to have a high percentage of fat (7.2% wet mass) as were the myctophids *Electrona antarctica* and *Gymnoscopelus nicholsi* (15.2 and 17.4% wet mass, respectively), which are consumed by Weddell seals along the Danco Coast and South Shetland Islands (Plotz, 1986; Casaux et al., 1997, 2006; Burns et al., 1998; Lake et al., 2003; Lenky et al., 2012). Although *P. antarcticum* is the most common prey species in many locations, it should be noted that not all Weddell seal prey species are high in fat; the lipid composition of most of the benthic *Trematomus* species investigated by Lenky et al. (2012) ranged between 0.5 to 4.6% wet mass (Burns et al., 1998; Lake et al., 2003; Casaux et al., 2006).

The diet of the Weddell seal is reflected in the metabolic phenotype of their skeletal muscles as determined by enzyme ratios. The citrate synthase (CS) to β-hydroxyacyl CoA dehydrogenase (HAD) ratio (CS:HAD), an indicator of how much

the skeletal muscle relies on lipid vs carbohydrates to fuel metabolism in both swimming and non-swimming skeletal muscle ranges between 0.1 and 0.4, suggesting a complete reliance on lipids for the generation of energy through aerobic metabolism (Reed et al., 1994; Kanatous et al., 2002, 2008). It is currently unknown if *in vivo* Weddell seal myotubes possess the same metabolic phenotype as the whole tissue from which they are derived and/or if they have any degree of metabolic flexibility—that is to say, the preferential utilization of fat oxidation during fasting, with a switch to glucose in the presence of insulin as described by Ukropcova et al. (2005). If the Weddell seal cells share the metabolic phenotype of the muscle tissue and are metabolically inflexible, we would expect them to need supplemental lipid for their growth and differentiation media.

We developed two methods that produced Weddell seal primary myoblasts and, in doing so, found that myoblasts would not proliferate after initial plating with standard media. We hypothesized that the growth and differentiation media of the myoblasts might require the addition of lipid, reflecting the high-fat diet of the Weddell seal. Based on the number of myotubes formed during differentiation, we conclude that the successful growth and differentiation media for Weddell seal cells is based on Dulbecco's modified eagles media (DMEM), supplemented with 2.5 to 5% lipid. Our results indicate that, when culturing non-model primary cells, researchers should consider the diet of the whole animal and tailor the culture media accordingly. These primary skeletal muscle cells will aid researchers in understanding the molecular regulation of the unique adaptations to diving in Weddell seal skeletal muscle.

Methods

Weddell Seal Primary Myoblast Isolation

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

The Colorado State University (CSU) Animal Care and Use Committee (ACUC) has approved all protocols used in this study (IACUC 07-1641A-01), and samples were collected under a permit from the National Marine Fisheries Service (MMPA #10751788-00). Myoblasts were isolated from an adult seal, juvenile seal, and seal pup captured in McMurdo Sound, Antarctica, in November 2006. The animal handling and biopsy protocol were performed as previously described; briefly, the Weddell seal was captured and kept calm using a head bag and chemically immobilized with an IV injection of Telazol (1 mg kg^{-1}) (Kanatous et al., 2008; Trumble et al., 2010). A local injection of Lidocaine (1 ml) was administered to the biopsy site, and the site was cleaned with Betadine. A small incision through the skin, blubber layer, and underlying fascia was made using a sterile #10 scalpel. The primary swimming muscle (*m. longissimus dorsi*) was biopsied with a sterile 6-mm biopsy cannula. Sterile forceps were used to transfer the muscle biopsy from the cannula. The biopsy was quickly disinfected in 70% ethanol and transferred into a 15-ml conical tube of Ham's F-10 media and placed on ice, for which care was taken so that the sample did not freeze. The biopsy was then transported from the field site via snowmobile to the Albert P. Crary Science and Engineering Center at McMurdo Station; the traverse took 30 min.

Once at the Crary laboratory, the biopsies were processed in one of two ways. The first method is as follows: The biopsy was transferred to a 15-ml conical tube containing 0.4 ml of collagenase-D (Boehringer Mannheim, reconstituted to 10 mg/ml with PBS and 5mM $CaCl_2$, product #1088-874) and dispase (Boehringer Mannheim, 2.4 u/ml, product #295-825) per 100 mg of tissue. The biopsy was left to incubate with the added enzymes for 7 min at 37° C to digest connective tissue. The sample was then transferred to a sterile 6 well plate, where ~2 ml of HAM's F-10 growth media (Hyclone Laboratories, Logan, UT, USA) were added, and the biopsy was further minced using a razor blade. The plate was then placed in an incubator (37° C, 21% O_2, 5% CO_2) for 1 h, after which the plate was removed from the incubator and observed for satellite cell migration under a microscope. Remaining pieces of tissue were then removed, and the plate was placed back in the incubator for 24 h. The cells were checked daily, and media was not removed from the dishes for 96 h post-plating to ensure cells had a change to adhere to the bottom of the plate. After the 96 h, when cells were observed growing in a dish, the media was changed daily. When the cells reached 40 to 60% confluence, they were passaged using trypsin. This method was tried on tissue from the adult male only and successfully produced cells.

The second method used during isolation is as follows: The biopsy was removed from the media in which it was transported and placed in a 60-mm cell culture dish where it was minced into 1 mm pieces using a razor blade. The plate was placed into an incubator (37° C, 21% O_2, 5% CO_2) for 1 h, after which the culture dishes were removed and observed. After 24 h, the muscle tissue was removed from the plate. The cells were checked daily, and fresh Ham's F-10 growth media was added as needed. At day 7, media was changed

from HAM's F-10 growth media to low glucose (1,000 mg/L) 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 d 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 min, the supernatant containing the myoblasts was removed and placed onto a new cell culture dish leaving the majority of the fibroblasts behind. This method successfully produced cells from a Weddell seal adult, juvenile, and pup.

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 CSU in Fort Collins, Colorado, where they are currently maintained and stored. At CSU, only cells from the adult male were unfrozen, proliferated, and differentiated.

During the initial culture of the cells in Antarctica, low glucose (1,000 mg/L) DMEM was used in the growth media, and no lipid was added. In these cases, using high (4,500 mg/L) glucose DMEM resulted in very poor to zero growth. When cells were unfrozen at our laboratory at CSU, they would not grow back in the low-glucose media. We, therefore, considered the diet of the whole animal and added lipid to the media. We found that myoblasts started to proliferate after adding at least 2.5% lipid (trials at 1% lipid produced very poor growth). After approximately 10 passages, cells began to do well in a high-glucose environment as long as lipid was present.

Media Recipes for Weddell Seal Skeletal Muscle Cells

Various growth and differentiation medias were made to test the potential effects on growth and differentiation of this unique primary cell line. The Weddell seal cells were grown and differentiated in their respective media supplemented with lipid throughout the experiment. Because our preliminary studies concluded that the cells did not grow in media without lipid supplementation and grew poorly in media supplemented with 1% lipid, no "control" media with 0% lipid was tested in order to avoid wasting these rare samples, and lipid concentrations higher than 1% were assessed. A commercially available 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 mixture, chemically defined by the manufacturer (Sigma Aldrich, St. Louis, MO, USA), is comprised of the following components: non-animal derived fatty acids (2 μg ml^{-1} arachadonic acid, and 10 μg ml^{-1} each of linoleic, linolenic, myristic, oleic, palmitic, and stearic acid), 0.22 mg ml^{-1} cholesterol from New Zealand sheep's wool, 2.2 mg ml^{-1} Tween-80, 70 μg ml^{-1} tocopherol acetate, and 100 mg ml^{-1} Pluronic F-68 solubilized in cell culture water. For the differentiation media, human recombinant insulin (Gibco, Grand Island, NY, USA) was in a stock solution of 4 mg/ml, and the bovine transferrin APO (Invitrogen, Carlsbad, CA, USA) was in a stock solution of 20 mg ml^{-1}. In this experiment, day 1 of differentiation was counted 24 h after growth media was replaced by differentiation media.

Growth Media

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

Differentiation Media

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

Myotube Count

After plating the cells on Petri dishes (100 × 20 mm), we proliferated the cells for 15 d to create enough plates for our experiments. While the proliferation period of any cell line is determined by the total number of plates needed, this proliferation time seems to be similar to that of mouse primary muscle cells (Krause et al., 2013). While our proliferation times were similar to that of other primary muscle cell culture experiments, in our experience, primary muscle cells take approximately twice as long to proliferate to the same magnitude as immortalized murine skeletal muscle cells (ATCC, Manassas, VA, USA). During that time, the confluence (plate coverage) was estimated daily, and cells were passaged. It is important to note that not all lipid concentrations were passaged at the same time as they did not reach appropriate confluence at the same time (plates were passaged at 75 to 100% confluence). For example, on day 4, myoblasts in the 7 and 10% lipid media condition were not passaged with the rest of the lipid conditions but, instead, passaged for the first time at day 8. Cells were monitored with a microscope, and photographs were taken using a Canon Powershot (Canon Inc., Lake Success, New York, USA) mounted to a Carl Zeiss Invertoskope (Carl Zeiss Inc., Thornwood, New York, USA) at 100× magnification for the following 7 d. Six plates of cells were grown in

each lipid treatment, and each was photographed once per day for the 7 d. A small circle was drawn onto the lid of each Petri dish with a Sharpie pen in an attempt to always photograph the same area of each plate; care was taken to not rotate the plate lid. The photographs were later analyzed with the Carl Zeiss Axio Vision program to count the number of myotubes. The photo range had a field of view of 270 μm × 2,037 μm. All 210 photos of the cells were analyzed. A 2 × 2 grid was created on the computer monitor to separate the photo into four 135 μm × 1,018.5 μm boxes. This allowed myotube counts to be efficient and precise, and ensured that each myotube was only counted once. The total number of myotubes were counted in each photograph and recorded.

Immunocytochemical Confirmation of Myotube Presence

Plates were probed for desmin and counterstained with DAPI to confirm the presence of multinucleated myotubes. Methods were based on Rosenblatt et al. (1995), with some modifications. Imaged cells were grown and differentiated on a 100 × 20 mm dish. All but 3 ml of media were removed from each plate and replaced with 3 ml of a 1:1 mixture of ice-cold acetone and methanol. Samples were incubated at 4° C for 10 min, followed by three, 10-min washes in PBS. Plates were then incubated in 5% horse serum in PBS for 45 min to reduce non-specific binding. Mouse anti-desmin primary antibody (BioGenex) was diluted to 1:100 in 5% equine serum, incubated for 1.5 h, and was followed by three 10 min PBS washes. DAB chromogen was used for detection after a 1.5 h incubation with 1:20 secondary antibody conjugated with horse-radish peroxidase. DAPI (300 nM) counter-stain was added for 10 min, after which images were taken.

Statistical Analysis

SigmaStat, Version 2.0 (Ashburn, VA, USA) was used for all statistical analyses. Analysis of variance (one-way ANOVA) with Tukey *post-hoc* tests was used ($p \leq 0.05$) for comparison of myotube numbers between lipid treatment groups each day. For the comparison of myotube counts between days 1 and 7 within a lipid treatment group, a one-way repeated measures ANOVA with a Tukey *post-hoc* test was utilized for statistical analysis ($p \leq 0.05$). After day 4, only the 2.5, 5, and 7% groups were subject to ANOVA between group. For all calculations, if data did not pass normality or equal variance tests, ANOVA on ranks was performed. The results in all figures are presented as means ± s.e.m.

Results

Isolation and Culture of Primary Weddell Seal Myoblasts

We identified two methods that produced myoblasts capable of proliferation and differentiation (Figure 1). We were able to harvest cells from one adult, one juvenile, and one pup but have, at this time, only differentiated cells from the adult male. One method utilized mechanical processing, while the second used both enzymatic and mechanical processing techniques. Samples were initially plated on HAM's F-10 but were moved to low-glucose DMEM-based media on day 7. Samples would only grow in low-glucose media until passage 10 when we were able to proliferate them in high glucose media. After being frozen down at -80° C with 100% isopropyl alcohol and subsequently placed in liquid nitrogen for storage, thawed cells were capable of proliferating again only with the addition of lipid to the growth media.

Myotube Count

After being thawed from the liquid nitrogen, Weddell seal cells did not proliferate in media not supplemented with lipid or supplemented with low (1%) amounts of lipid. When cultured in media supplemented with 2.5 and 5% lipid, the number of Weddell seal myotubes significantly increased between day 1 (24 h after the initiation of differentiation) and day 7 (168 h) of differentiation ($n = 6$; $p = 0.027$ and $p < 0.001$, respectively; Figure 2). Taking into account the average myotube counts between days 1 and 7, the 2.5% lipid treatment group grew at a rate of 10.6 myotubes/d, the 5.0% group grew at 11.3 myotubes/d, the 7% group grew at 4.1 myotubes/d, and the 10% grew at -5.4 myotubes/d; there was no statistical significance between the 2.5, 5, and 7% growth rates. All groups had statistically greater rates than the 10% treatment group because almost all myotubes in that cohort died by day 4 of the trial ($p < 0.05$). There was a significant difference between the 2.5 and 10%, and 5 and 10% treatment groups only on days 2 and 3 of differentiation ($p < 0.05$ for both) as well as between 2.5 and 7% on day 5 of differentiation ($p = 0.042$; Figure 2). The presence of myotubes was confirmed via DAPI and desmin staining (Figure 3). Images of myotubes on days 2, 4, and 7 of differentiation can be found in Figure 4. Starting on day 1, the number of myotubes in 10% differentiation media decreased until zero myotubes were found on day 5, indicating high myotube mortality (Figure 2). These results support the idea that high lipid content can be cytotoxic, although it is interesting to note that a high lipid concentration did not affect an

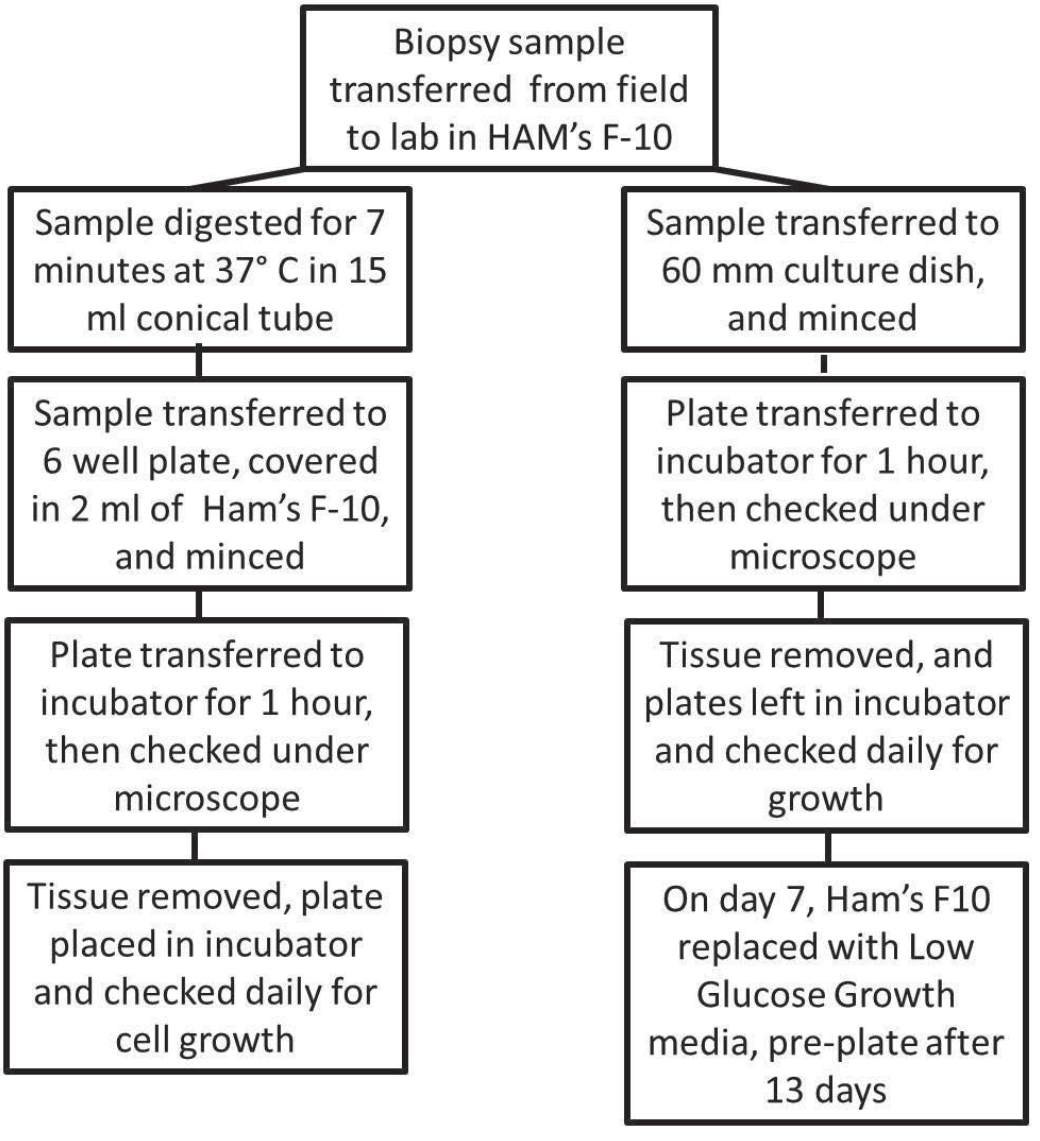

Figure 1. Flow chart of two methods that produced primary Weddell seal (*Leptonychotes weddellii*) skeletal muscle cells; one method (left) utilized mechanical and enzymatic processing methods, and the other (right) only relied on mechanical processing methods.

undifferentiated myoblast as dramatically. The cytotoxicity of lipid has mostly been studied in cancerous cell culture lines, and, as reviewed by Noding et al. (1998), it is thought that the cytotoxicity of polyunsaturated fatty acids in certain cell culture lines may be due to the products that result after their oxidation and may be affected by the ingredients in the cell culture media. While our primary cells are not immortalized or cancerous, their demise may be the result of similar mechanisms.

Discussion

The cells supplemented with 2.5 and 5% lipid during proliferation and differentiation showed a significant increase in myotube number between the first (24 h after the start of differentiation) and last day of differentiation. Given this result, we suggest that the most successful growth and differentiation medias for Weddell seal skeletal muscle cells contain between 2.5 and 5% lipid. It is interesting to note that the myotube count

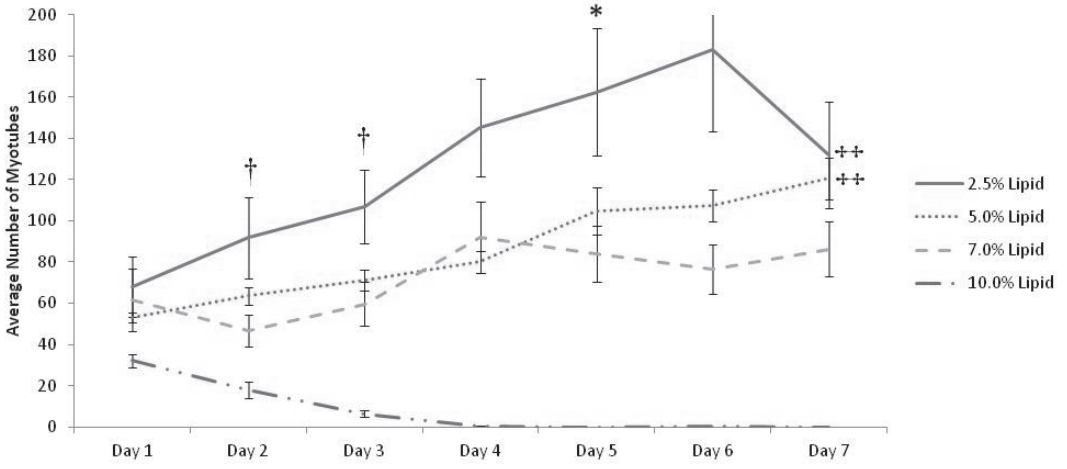

Figure 2. Myotube counts from day 1 of differentiation to day 7. Significant differences between days 1 and 7 within one lipid treatment group indicated by ‡; significant difference between 2.5 and 7% denoted by *; significant differences between *both* 2.5 and 10 and 5.0 and 10 indicated by †; significant difference between days 1 and 7 within lipid treatment groups was determined at $p \leq 0.05$, one-way ANOVA with repeated measures; and significant difference between treatment groups for each day was determined by one-way ANOVA. Data are presented as means ± s.e.m.

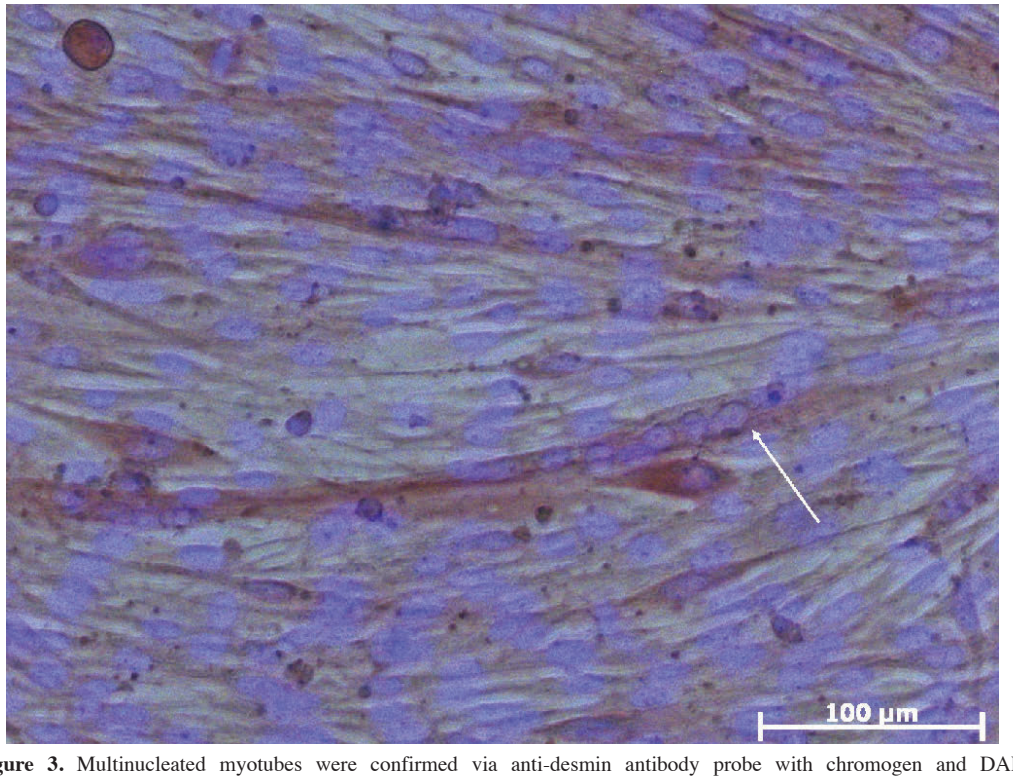

Figure 3. Multinucleated myotubes were confirmed via anti-desmin antibody probe with chromogen and DAPI counter-staining. The arrow points to multinucleated myotube, 200× magnification.

in the 2.5% media treatment groups dropped after day 6; a possible cause for this decrease is that these cells matured to the point of having functional contractile units earlier than the other treatments as contraction is one cause of myotube detachment in plates (Cooper et al., 2004). This

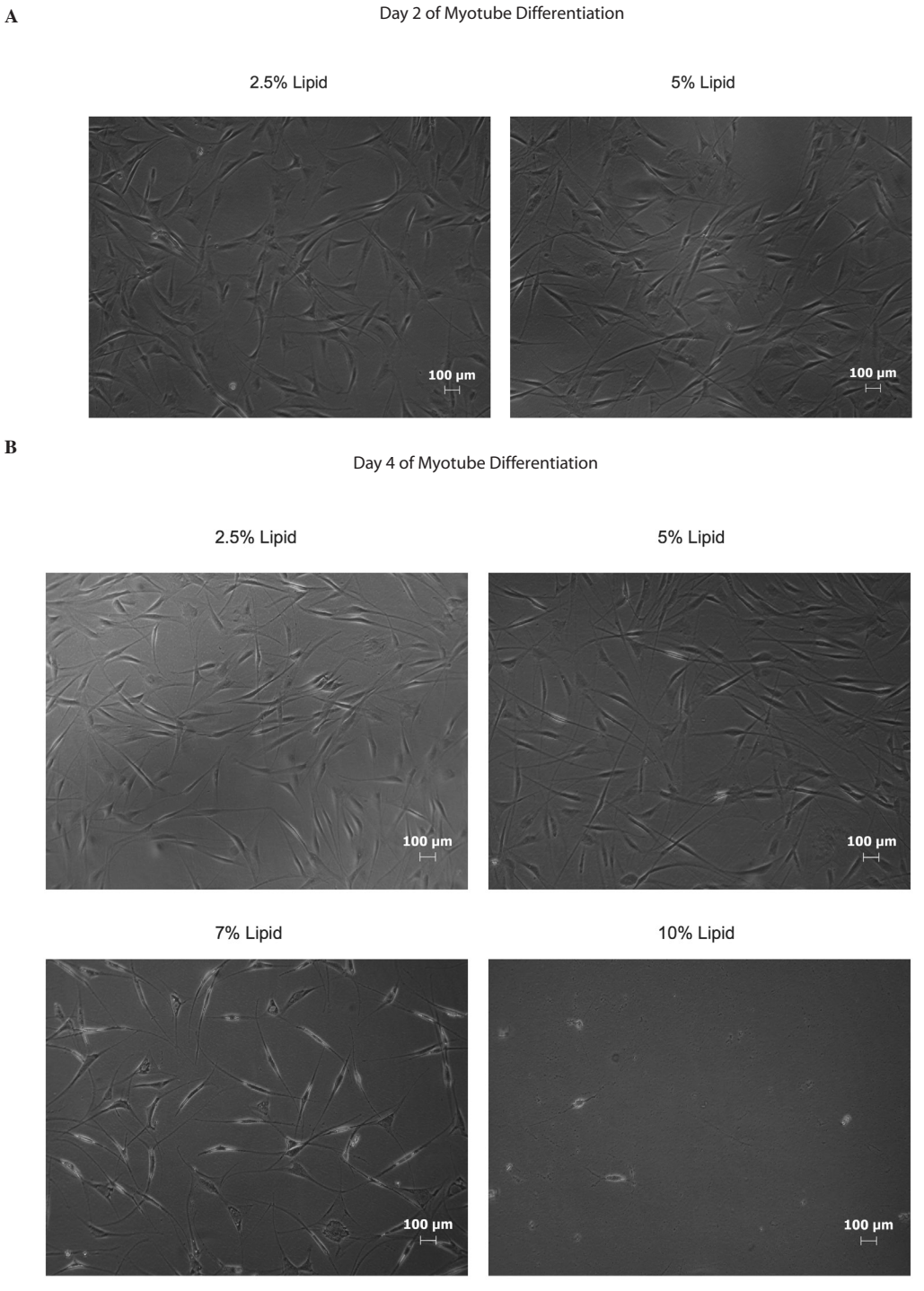

Figure 4A & B. Myotubes in different lipid treatment groups on days 2, 4, and 7 of differentiation: (A) 100× magnification of Weddell seal skeletal muscle myotubes after 2 d of differentiation; and (B) 100× magnification of Weddell seal skeletal muscle myotubes after 4 d of differentiation. Scale bars = 100 µm.

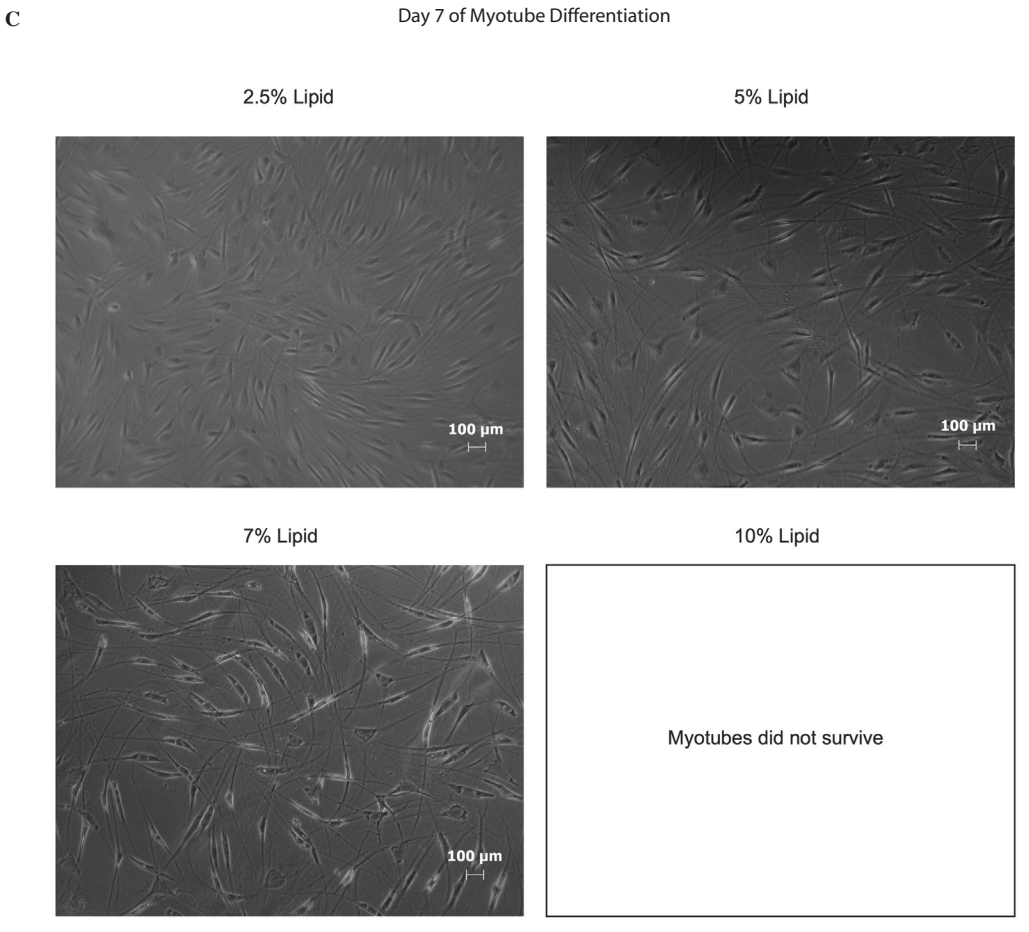

Figure 4C. Myotubes in different lipid treatment groups on days 2, 4, and 7 of differentiation: (C) 100× magnification of Weddell seal skeletal muscle myotubes after 7 d of differentiation. Note the absence of an image for the 10% lipid mixture group. Scale bars = 100 µm.

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

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

In the laboratory, the diet of the average rodent can range between 4.7 to 5% fat (wet weight, although these pelleted diets are low in moisture), with high-fat diets ranging between 18 and 35% fat (pelleted diet calculated "as is," ~3.5% moisture) (LabDiet, Certified Rodent Diet, product #5002; Harlan

Laboratories, Teklad Certified Rodent Diet, product #8728C; Harlan Laboratories, Teklad Custom Research Diet: Diet Induced Obesity Diets, products #TD.95217 and #TD.03584). It is not surprising that the percent of fat in the standard diet of these rodents is not much less than that of the favored Weddell seal prey *P. antarcticum* and can ostensibly be higher. This flexibility seems to be shared by their cultured skeletal muscle cells; unpublished data from our lab have shown that immortalized mouse skeletal muscle cells (c2c12 ATCC, product #CRL-1772) can proliferate and differentiate in media with a range of glucose concentrations (1,000 to 4,500 mg/L), and in the presence of between 2.5 to 5% lipid (Schlater et al., 2014). While the *in vitro* metabolic flexibility of c2c12 muscle cells in relation to that of *in vivo* mouse muscles has not been compared, studies by Ukropcova et al. (2005) in human skeletal muscle cells seem to suggest that there is a correlation between the *in vivo* metabolic flexibility of whole tissue and *in vitro* measures of the metabolic flexibility of cultured cells. The authors found that, in healthy humans, the *in vitro* adaptability (the increase of fat oxidation with increase in high palmitate concentration) of skeletal muscle myotubes is correlated with the *in vivo* metabolic flexibility of the muscle from which they were cultured. However, comparisons of *in vitro* suppression, defined by the authors as the ability of glucose to suppress fat oxidization in the absence of insulin, was compared with an *in vivo* cohort subject to a euglycemic, hyperinsulinemic clamp, so direct comparisons of the ability to switch from fat oxidation to glucose are unclear. Additionally, Ukropcova et al. (2005) measured variability within one species, suggesting that broad statements about the relationship between whole tissue and cell muscle metabolic flexibility within a species cannot be made without first characterizing the intraspecies range of this relationship. Thus, while the metabolic phenotype and flexibility of skeletal muscle may have an intrinsic component, the nature of this contribution is uncertain.

It is possible that this lipid requirement of Weddell seal primary skeletal muscle cell culture is reflective of the lipid-based metabolic phenotype indicated by CS:HAD ratios in the whole muscle tissue from which the cells were biopsied, which, in turn, is reflective of the Weddell seals' diets. Alternatively, it is also possible that the cells' lipid requirement is not imposed by a lipid-fueled aerobic metabolic phenotype but is instead reflective of the role of lipid in regulatory myoglobin pathways. New evidence suggests that lipid either directly or indirectly increases the production of myoglobin in both c2c12 and Weddell seal skeletal muscle culture (De Miranda et al., 2012; Schlater et al., 2014).

De Miranda et al. (2012) found that Weddell seal cells grown in normoxic conditions demonstrate increased myoglobin expression with higher lipid media concentrations (2.5 vs 5%). Prior to this result, increases in myoglobin were seen in the presence of hypoxia and a second stimulus, such as exercise, or after fiber-type changes (as reviewed in Kanatous & Mammen, 2010). In addition to its oxygen storage properties, myoglobin has the ability to scavenge reactive oxygen species (ROS). Schlater et al. (2014) found that the addition of a ROS scavenger to culture media supplemented with lipid reduces a lipid-induced myoglobin increase in normoxic c2c12 cells. Both of these results are suggestive of a yet uncharacterized lipid-based myoglobin regulatory pathway and, in the case of the terrestrial c2c12 cells, one involved in ROS mitigation. It is possible that cultured Weddell seal skeletal muscle cells cannot proliferate without a lipid-stimulated expression of myoglobin; whether this would be due to the ROS scavenging or oxygen storage properties of myoglobin is unclear. Because the cells grown in normoxic environments exhibit this increase, a ROS-scavenging role in these cells is possible. Future studies in our laboratory will focus on unraveling the nature of these pathways.

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

Author Contributions

TMG synthesized data and methods, prepared the manuscript, and performed ICC staining; TLG contributed significantly to cell culture, media

development, and preparation of the manuscript; LEP contributed to the primary culture plating protocol in Antarctica and preparation of the manuscript; MAD contributed to cell culture, media development, and preparation of the manuscript; LMC and AML contributed to the cell culture; and SBK contributed to the initial primary culture plating protocol, cell culture, media development, and preparation of this manuscript.

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