Fiber type, intramyocellular lipid and mitochondrial mass in human primary myotubes retain the metabolic characteristics of the donor

Dissertation for the degree of Master of Pharmacy

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Oslo, May 2011
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Abstract

Fiber type composition is a significant and critical characteristic of skeletal muscle. The purpose of this study was to investigate the differences in metabolic characteristics in athletes, lean and T2DM determined in human primary myotubes, and to compare metabolic characteristics such as fiber type composition, lipid content, mitochondrial capacity, physical fitness and activity measured in previous studies in vivo versus fiber type-I and lipid content measured in primary myotubes cultured from the same donors. Moreover, we determined the effect of progressive passaging of myotubes on fiber type-I content, lipid content and mitochondrial mass.

Six athletes, 6 sedentary lean and 6 subjects with T2DM were studied. Muscle samples were obtained from Vastus Lateralis (VL) from which myoblasts were sorted, cultured and differentiated into myotubes. Type-I fiber and lipid content were quantified by immunohistochemistry technique using MHC-I antibody and bodipy respectively. Mitochondrial mass was measured by Mitotracker Green.

Myotubes from athletes had a significant higher amount of fiber type-I (9.9±1.8 vs. 1.6±1.0 vs. 2.0±0.8 AU; p<0.001), lipid content (0.047±0.003 vs. 0.032±0.001 vs. 0.033±0.001 AU; p<0.001) and mitochondrial mass (105.2±3.8 vs. 80.0±3.0 vs. 90.0±3.9 AU; p<0.01) compared to myotubes cultured from lean and T2DM subjects; fiber type-I measured in myotubes correlated with fiber type-I measured directly in tissue and also with mitochondrial capacity measured as ATPmax. Lipid content measured in myotubes was not correlated with intramyocellular lipid (IMCL) measured in tissue. However, lipid measured in myotubes strongly correlated with insulin sensitivity measured by hyperinsulinemic euglycemic clamp (r²=0.29; p<0.05), physical fitness, measured by VO2max (r²=0.54; p<0.05) and physical activity level (PAL) (r²=0.85; p<0.01). No significant passage effect on fiber type-I content, lipid content and mitochondrial mass was observed in myotubes through passage 4 to passage 7. However, a decreasing trend was observed in fiber type-I (P=0.14) and mitochondrial mass (P=0.05) with multiple passaging.
Abstract

In conclusion, primary myotubes retain fiber type characteristics of skeletal muscle *in vivo*. Lipid measured in cultured myotubes, although not associated with IMCL content measured in tissue, it was strongly associated with insulin sensitivity and physical fitness and activity of the donor.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Akt</td>
<td>Non-specific serine/threonine-protein kinase family</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine 5’-monophosphate (AMP)- activated protein kinase</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C-zeta/lambda</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary unit</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchinonic assay</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>Bodipy</td>
<td>Boron-dipyrromethene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-enzyme A</td>
</tr>
<tr>
<td>CPT-I</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CPT-II</td>
<td>Carnitine palmitoyltransferase II</td>
</tr>
<tr>
<td>CRAT</td>
<td>Carnitine acetyltransferase</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium (low glucose with L-glutamin)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EMBS</td>
<td>Estimated metabolic body size</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>Fatty acid translocase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GDR</td>
<td>Glucose disposal rate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HMIT</td>
<td>H⁺-coupled myo-inositol transporter</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>Insulin-like growth factor binding protein-2</td>
</tr>
<tr>
<td>IMCL</td>
<td>Intramyocellular lipid</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>LMM</td>
<td>Linear mixed model</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media (with Glutamax)</td>
</tr>
<tr>
<td>MET</td>
<td>Metabolic equivalent task</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>P38MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PAL</td>
<td>Physical activity level</td>
</tr>
<tr>
<td>PBRC</td>
<td>Pennington Biomedical Research Center</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDK-1</td>
<td>3-phosphoinositol dependent protein kinase-1</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor γ co-activator 1α</td>
</tr>
<tr>
<td>PGC-1β</td>
<td>Peroxisome proliferator-activated receptor γ co-activator 1β</td>
</tr>
<tr>
<td>PI(3,4,5)P₃</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PI(3,4)P₂</td>
<td>Phosphatidylinositol (3,4)-bisphosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmuno precipitation assay</td>
</tr>
<tr>
<td>RMR</td>
<td>Resting metabolic rate</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBS</td>
<td>Trisbuffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Trisbuffered saline with Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TDEE</td>
<td>Totally daily energy expenditure</td>
</tr>
<tr>
<td>TNS</td>
<td>Trypsin neutralizing solution</td>
</tr>
<tr>
<td>VL</td>
<td><em>Vastus Lateralis</em></td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$</td>
<td>Maximal oxygen uptake/consumption</td>
</tr>
</tbody>
</table>
# Introduction

## 1.1 Type 2 diabetes mellitus

Diabetes is a chronic disease that affects more than 220 million people in the world [1]. Diabetes is defined as "A metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both" [2].

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia, where insulin resistance and β-cells dysfunction are thought to be the main cause of the increased glucose level in blood. Elevated blood glucose primarily affects the skeletal muscle, fat tissue and liver, which can cause dyslipidemia (increased very low density lipoprotein (VLDL) and triglyceride and decreased high density lipoprotein (HDL)) [3], lipotoxicity and glucose toxicity in these metabolic organs [4]. Other co-morbidities associated with T2DM are hypertension and obesity, which increase the risk of cardiovascular diseases (CVD) and insulin resistance [3, 5].

Lifestyle interventions and pharmacotherapy are used to treat T2DM and the co-morbidities associated with T2DM. Drug substances that are used to control the elevated blood glucose have different mechanisms that either enhances insulin sensitivity (thiazolidinediones), decreases hepatic glucose output (metformin), reduces glycemic excursions (α-glucosidase inhibitors) or insulin secretion enhancers (sulfonylurea derivates). To prevent the co-morbidities associated with T2DM, it is important to start interventions (lifestyles changes and pharmacotherapy) in an early phase [4].

Despite the pharmacotherapy and lifestyle interventions, the rise of number of people with T2DM is estimated to be 336 million in 2030 [6]. Investigations of new treatments are therefore useful and important to prevent this growing disease. The majority of these drug investigations are performed in vitro using primary cell culture models. It is, therefore, critical that in vitro cell culture methods used for these purpose, possess similar metabolic characteristics of the donors measured in vivo. Type-I fiber
composition plays a critical role in determination of skeletal muscle metabolic characteristics due to its highly oxidative properties (compared to glycolytic type-II fibers). To the best of our knowledge, there are no studies that have identified type-I fibers in human primary myotubes and investigated whether fiber type characteristics in primary cell culture models are comparable to fiber types measured in vivo using skeletal muscle biopsy.

1.2 Skeletal muscle

Skeletal muscle is an important source for energy metabolism and make up approximately 55% of the body mass [7]. For healthy individuals, the skeletal muscle is responsible for about 80% of whole-body insulin-stimulated glucose uptake. The glucose uptake could be reduced to 40% in lean individuals with insulin resistant [8].

1.2.1 Glucose metabolism in skeletal muscle

Skeletal muscle is the primary tissue responsible for the glucose disposal [8], whereas the glucose transporter proteins (GLUT) have an essential role in regulating the glucose transport. 13 isoforms of GLUT have been identified in the human genome, respectively GLUT1 to GLUT12 and H+-coupled myo-inositol transporter (HMIT). The isoforms differ in kinetic properties and have distinctive substrate specificities in which muscle/adipose glucose transporter (GLUT 4) and erythocyte/hHepG2/brain glucose transporter (GLUT1) contribute to most of the glucose uptake in skeletal muscle [9]. These two isoforms are highly important to maintain glucose homeostasis in the body and the amount of these proteins plays a critical role in the glucose metabolism. In 1990 Kern et al. demonstrated that the level of GLUT4 transporter protein was a major determinant in the ability of skeletal muscle to respond to insulin [10]. GLUT4 protein determines the amount of intracellular glucose, as it favors the uptake of glucose into the cell [11]. They have also shown that GLUT4 protein is more abundant in red skeletal muscle (type I fiber and type IIa fiber) compared to white skeletal muscle (type IIb) [10].
GLUT4 protein is mainly stimulated/translocated by two different factors, insulin and muscle contraction (exercise) as illustrated in figure 1.1. When insulin binds to the extracellular α-subunit of insulin receptor, it leads to an autophosphorylation of the transmembrane β-subunits and activation of insulin receptor substrate (IRS) family. This subsequently activates phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P₂) and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P₃) which in turn activates 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and signal to Akt and atypical protein kinase C-zeta/lambda (aPKC). This signal cascade favors translocation of GLUT4 to the cell membrane [12]. When insulin binds to the insulin receptor p38 mitogen-activated protein kinase (p38MAPK) is also activated, which is further responsible for the activation of GLUT4. GLUT4 takes up glucose into the muscle cell and transforms to glucose 6-phosphate by phosphorylation. Glucose 6-phosphate can either be stored as glycogen or be used as a fuel source in glycolysis [11].

As mentioned above, exercise also stimulates the translocation of GLUT4 protein (figure 1.1). Adenosine 5´monophosphate-activated protein kinase (AMPK) is an important enzyme that plays a critical role in regulation of this translocation process. When the muscle contracts, GLUT4 can be translocated by three mechanisms, respectively AMPK translocates GLUT4 directly to the cell surface, AMPK stimulates p38AMPK to activate GLUT4, and/or muscle contraction activates an AMPK-independent pathway which leads to a translocation of GLUT4 to the T-tubulus [12].
Introduction

Figure 1.1: Glucose metabolism in skeletal muscle stimulated by insulin and muscle contraction. IRS, insulin receptor substrate; PI(3,4)P₂, Phosphatidylinositol (3,4)-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol (3,4,5)-trisphosphate; PDK-1, 3-phosphoinositide dependent protein kinase-1; aPKC, atypical protein kinase C-zeta/lambda; GLUT4, glucose transporter; AMPK, adenosine 5’monophosphate-activated protein kinase; p38MAPK, p38 mitogen-activated protein kinase. Modified from F. Trembley et al. (2003) [12].

1.2.2 Lipid metabolism in skeletal muscle

Free fatty acids (FFAs) translocate into the muscle cells either by specific plasma membrane fatty acid binding protein (FABP), fatty acid translocase (FAT/CD36), or to a minor extent by diffusion depending on the size of the FFA molecule (figure 1.2). Inside the cytosol, the FFA is converted to acyl-CoA by acyl-CoA synthetase enzymes or thio kinase. The acyl-CoA complex can either be used for intramyocellular lipid (IMCL) synthesis or it can bind to carnitine. Binding to carnitine demands a contribution from the enzyme carnitine palmitoyltransferase I (CPT I) located on the outside of the outer mitochondrial membrane, and the binding releases free CoA. This is the first step transporting FFA in to the mitochondria. The second step includes translocation of the acyl-carnitine complex over the matrix by a carnitine acetyltransferase (CRAT) and then a reconversion to acyl-CoA by the enzyme carnitine palmitoyltransferase II (CPT II) located on the inner membrane of the mitochondria. Acyl-CoA is further exposed for β-oxidation resulting in acetyl-CoA and can then join the tricarboxylic acid cycle (TCA) [13, 14].
1.2.3 Insulin resistance in skeletal muscle

Insulin is an endogenous hormone with various metabolic functions including amino acid uptake, protein synthesis, proteolysis, adipose tissue triacylglycerol lipolyses, lipoprotein lipase activity, VLDL triacylglycerol secretion, muscle and adipose tissue glucose uptake, muscle and liver glycogen synthesis, and endogenous glucose production [15]. When normal level of insulin fails to inhibit an increase in plasma FFA in muscle cells, it is known as insulin resistance [16]. Insulin resistance in skeletal muscle occurs primarily due to this increase in FFA and lipid metabolites such as long chain fatty acyl-CoA, diacylglycerol (DAG) and ceramides that disrupt insulin
mediated glucose uptake pathway [17]. For example, higher DAG content in skeletal muscle activates serine/threonine phosphorylation, which in turn leads to a reduction in the glucose uptake via the IRS-1 pathway. Ceramide inhibits the signaling protein Akt, which interferes with the insulin signaling pathway resulting in reduced glucose uptake [16]. Elevated levels of IMCL have been associated with insulin resistance in both obesity and T2DM due to accumulation of lipid metabolites such as DAG and ceramides [18]. It has also been shown that the lipid oxidation in the muscle is reduced in T2DM [19], and it is thought that this is affected by mitochondrial dysfunction as mitochondria are the main site for the fatty acid oxidation. Another source that can be responsible for the increased supply of fatty acid to the skeletal muscle is a dysfunction in the adipose tissue [20].

However, recent studies suggest that populations such as endurance trained athletes are associated with a high insulin sensitivity even though IMCL deposit is elevated in the skeletal muscle [16, 21, 22]. In these subjects it is proposed that IMCL protects the skeletal muscle from lipotoxicity caused by ceramides and DAG accumulation, also described as “The athlete paradox” [16, 23]. This suggestion is supported by 1) size of the IMCL within the lipid droplets and 2) the lipid-to-mitochondria contact. It is thought that a decrease in lipid droplet size, hence an increase in lipid droplet contact to mitochondria [16, 18] results in an enhanced oxidative capacity and IMCL turnover in athletes [23]. Therefore, lipid metabolites such as ceramide and DAG are prevented to accumulate and cause insulin resistance [16, 24]. Although the relationship between IMCL and insulin sensitivity is still not completely clear, increased IMCL, especially in sedentary and T2DM, may cause harmful consequences and complications [16].

1.2.4 Skeletal muscle and T2DM

A defect in mitochondria, or mitochondrial dysfunction, has been suggested to cause insulin resistance [25, 26]. In 2004 Petersen et al. showed that a defect in mitochondrial oxidative phosphorylation caused dysregulation in intracellular fatty acid metabolism, hence increased IMCL in T2DM. The study postulated that the amount of fiber type-I causes a reduction in mitochondrial content and thereby causes defect in mitochondrial oxidative phosphorylation, which leads to insulin resistance in the
skeletal muscle [27]. On the other hand, Bajpeyi et al. demonstrated that mitochondrial dysfunction is not “causally” related to insulin resistance in the T2DM population. Results did not show any correlation between maximal mitochondrial capacity ($ATP_{\text{max}}$) and insulin sensitivity, hence mitochondrial capacity is not associated with insulin action in T2DM [28]. This finding is also supported by Toledo et al. which suggested that training improves insulin sensitivity without changing mitochondria content [29]. Nevertheless, fiber type composition is a distinguished characteristic of skeletal muscle which depends on the muscle group being studied and also plays an important role in determine IMCL content [30].

1.3 Fiber type

Skeletal muscle is a heterogeneous organ consisting of long and multinucleate muscle fibers. By using histochemical methods, fiber type in skeletal muscle can be identified as myosin heavy chain (MHC) type I, type IIa and type IIb. Type I fiber has lower concentration of myofibrillar-ATPase (M-ATPase reaction) and is also referred as “slow twitch”, whereas type II fiber has higher concentration of myofibrillar-ATPase, and is referred as “fast-twitch” [31]. In 1994 Smerdu et al. demonstrated another human skeletal muscle gene IIx MHC, which is more abundant in type IIb fiber [32].

1.3.1 Fiber type-I

*Vastus Lateralis* muscle consists of ~41% fiber type-I [33]. Fiber type-I has a high oxidative capacity compared to fiber type-II. It is also found that the capillary density, lipid storage capacity, insulin binding, insulin stimulated glucose uptake and GLUT4 are higher in type-I fiber [34]. Thus, the glucose uptake and metabolism, and insulin sensitivity in type-I fibers are greater [35]. Billeter et al. showed that type-I fibers contained slow myosin (MHC-I) only, in most of the human type-I, although some species had both slow and fast myosin [36]. Myosin is a protein, which is important for the molecular motor of the muscle contraction when being active [37]. Type-I fiber is thus more abundant in endurance trained subjects [35] and is important in the
metabolism of fatty acids and glucose. However, sprinters show more of fiber type-II [38].

A study on mitochondrial function, performed by Parikh et al. showed that type-I fibers were positively correlated with 15 oxidative phosphorylation (OXPHOS) genes, particularly NDUFB5 and ATP5C1 genes. These genes are affected by aging (decreases with age) and exercise training (increases with training), and reflect the mitochondrial function since the mitochondrial ATP production is related to maximal oxygen consumption (VO2max) [39].

1.3.2 Fiber type-II

Type-II fibers are mainly divided into two distinctive groups, respectively type IIa and type IIb [40]. Type-IIa fibers are considered more oxidative compared to type-IIb fibers and resemble the type-I fibers [35]. On the other hand, type-IIb fibers are more glycolytic in nature. In Vastus Lateralis in human muscle, type-IIa is twice as common as type-IIb [35]. Studies have shown that the glycolytic enzymes phosphofructokinase (PFK) and glycogen phosphorylase have higher activity in type-II fibers, respectively type-IIb fibers [35, 41]. PFK is thought to bind to cellular compartments such as mitochondria, however, where in the mitochondria are not clarified. PFK is also highly significant in the regulation of glycolytic energy metabolism because of its allosteric behavior [42]. Both PFK and glycogen phosphorylase are important to sustain aerobic and anaerobic metabolism [35]. Thus, type-II fibers depend primarily on glycolytic pathways for energy production [35]. Mitochondrial content is also decreased in type-II fibers and has lower gene expression of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β) that regulate the mitochondrial biogenesis [43]. Stuart et al. demonstrated higher expression of the fructose transporter GLUT5 in type-II fibers compared to type-I fibers, which expressed higher level of GLUT4. GLUT5 has no ability to transport glucose, which results in a decreased glucose uptake in type-II fibers and reduced insulin sensitivity [44]. Data show that fiber type-I is more insulin sensitive compared to fiber type-II [10, 34, 45], but it is still unclear whether it is the difference in GLUT
transporters or other factors in the insulin-signaling pathway that causes the variation in fiber type.

1.3.3 Fiber type composition in lean, athletes and T2DM

It is well known that there may be a difference in fiber type and metabolic conditions in lean sedentary subjects compared to obese and/or T2DM subjects. Studies have shown that lean subjects have a significant higher percentage of type-I fibers and a lower percentage of type-II fibers compared to obese subjects [40, 46]. Mitochondria in skeletal muscle of lean sedentary subjects also seem to be bigger in size compared to T2DM [47].

Endurance trained athletes have higher proportion of fiber type-I and type-Ila compared to sedentary lean individuals [48]. Studies also show that the succinate dehydrogenase (SDH), which is an important oxidative enzyme [49], is higher in athletes [48]. Since type-I fiber consists of more mitochondria than type-IIb fiber, the oxidative capacity in athletes is greater than untrained subjects. Goodpaster et al. also demonstrated these findings in which trained subjects showed a significant higher IMCL content in skeletal muscle than lean untrained subjects, but similar to the T2DM subjects. However, SDH was 65% higher in trained subjects compared to the obese group, and 50% higher than the T2DM subjects [23].

In the past decade, many studies have also been performed on fiber types related to T2DM. Results have been controversial, where many of the data presented indicate an increased amount of type-II fibers, particularly type-IIb, and decreased amount of type-I fibers [34, 50-52]. On the other hand, some have also shown that there are no differences in fiber type between T2DM and normal control subjects [23, 53], but still a difference in the insulin-stimulated glucose transport, i.e. the insulin-stimulated glucose transport is reduced in T2DM when hyperglycemia occurs [53]. Despite this disagreement in fiber type composition, others have illustrated an impaired bioenergetic capacity of the skeletal muscle mitochondria in T2DM [47]. This was due to a lower activity of NADH:O2 oxidoreductase and citrate synthase activity, in addition to smaller mitochondria in T2DM subjects as mentioned earlier [47]. By using proton (1H) magnetic resonance spectroscopy techniques, Petersen et al. have found a
Introduction

significant increase in IMCL content in offspring of patients with T2DM, due to a decrease in mitochondrial phosphorylation by 30%. It is therefore hypothesized that this dysregulation of intramyocellular lipid metabolism is associated with insulin resistance in the skeletal muscle [27]. Table 1.1 shows a summary of each cohort’s distinctive metabolic characteristics.

Table 1.1: Summary of fiber type and metabolic characteristics of athletes, lean and T2DM subjects. T2DM, type 2 diabetes mellitus; VL, Vastus Lateralis; SDH, succinate dehydrogenase; IMCL, intramyocellular lipid.

<table>
<thead>
<tr>
<th></th>
<th>Athletes</th>
<th>Lean</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fiber type</strong></td>
<td>More of fiber type I and type IIa compared to untrained subjects [48]</td>
<td>Higher percentage of type-I fibers and a lower percentage of type-II fibers compared to obese subjects [40,46]</td>
<td>Higher amount of type IIB and lower amount of type I [30,50-52]</td>
</tr>
<tr>
<td><strong>Mitochondria and oxidative capacity</strong></td>
<td>Higher oxidative capacity (increased SDH) [23,48]</td>
<td>Mitochondria is bigger in size compared to T2DM [47]</td>
<td>Smaller mitochondria and lower oxidative activity (reduced NADH-O2 oxidoreductase, citrate synthase) [47]</td>
</tr>
<tr>
<td><strong>Intramyocellular lipid</strong></td>
<td>Higher IMCL in VL (The athlete paradox) [23]</td>
<td>Lower amount of IMCL compared to athletes, but similar to T2DM [23,27]</td>
<td>Higher amount of IMCL compared to non-diabetic [27]</td>
</tr>
</tbody>
</table>

However, despite the research performed on muscle tissue (in vivo) among athletes, lean and T2DM cohorts, data is limited when regarding the importance of in vitro primary cell culture in the investigation of metabolic characteristics and further research within T2DM and anti-diabetic drugs. Nevertheless, very little is known about the fiber type composition properties of humans in primary myotubes among these groups. It is therefore essential to determine if primary myotubes in cell culture model hold similar properties to represent different metabolic cohorts, respectively athletes, lean and T2DM.

1.4 Cell culture as a model

Cell culture models of human skeletal muscles are used in several research studies [54-56]. After obtaining a biopsy from human skeletal muscle tissue (e.g. Vastus Lateralis), cells are grown in culture media (figure 1.3). Cell cultures can be subcultured, and
depending on how many times it is subcultured, it represents the passage number e.g. passage 5 has been subcultured five times [57]. When cell cultures reach confluence, these can be brought to the next passage [58].

![Schematic sketch of primary skeletal muscle cell culture: from biopsy to myotubes and to different passages.](image)

Cell culture model is of great importance as this *in vitro* model provides an enormous platform to test scientific hypotheses by manipulation of cells metabolically as well as with drugs/stimulants. In proper environments, cells grow and differentiate robustly. Berggren et al. have also shown that only a little amount (~ 50 to 100mg) of human skeletal muscle is needed from biopsy or surgery to be able to grow skeletal muscle cells. When differentiating the cells from myoblasts to multinucleated myotubes (myogenesis), metabolic characteristics such as glycogen synthesis and insul-
Introduction

stimulated glucose transport, and contractile characteristics of skeletal muscle are preserved [58]. Differentiating of myoblasts is therefore an important process which also increases expression of proteins such as myosin and MHC [58]. However, it is still not known whether all of the genetic information is transferred from one passage to another and if different passages can be used in the same experiment.

Machida et al. performed a study on satellite cells from primary rat muscles in which cells were grown in three passages. Immunohistochemical data showed a decrease in the myogenic markers MyoD and desmin from passage one to passage three. The percentage of nuclei in myotubes was also reduced after the third passage [59]. Another study performed on satellite cells from pigs, showed a great diminished capacity for proliferation and myotube formation when cells were grown from the fifth passage to the seventh passage. This was demonstrated by detecting Insulin-like growth factor binding Protein-2 (IGFBP-2) which increases with depressed cell proliferation and myotube formation [60]. These findings demonstrate an alternation in cell proliferation through multiple passages, which explains why validation of primary cell culture model needs to be performed regularly. However, to the best of our knowledge, the effect of progressive passing on type-I fiber in myotubes grown from human skeletal muscle is still not known.

The fiber type composition in human skeletal muscle is an important and critical characteristic, which strongly associates with metabolic profiles such as IMCL, mitochondrial content and function of the skeletal muscle. In vitro experiments performed in primary myotubes cultured from skeletal muscle are of great significance, since metabolic conditions can be manipulated to understand metabolic pathways, functions and various physiological responses to drugs, substrate and metabolic stimuli [56, 61]. By using primary myotubes from distinctive cohorts such as athletes, lean and T2DM subjects, metabolic features like insulin sensitivity and oxidative capacity of skeletal muscle can be determined. It is therefore essential to clarify if primary myotubes in cell culture models hold similar properties to represent different metabolic cohorts.
1.5 Aims

This study was designed to determine

1) Whether fiber type composition determined in human primary myotubes is comparable with fiber type composition determined directly in skeletal muscle frozen tissue
2) Whether fiber type composition properties are retained in a primary cell culture model with increasing passage numbers
3) Whether metabolic components such as lipid and mitochondrial content measured in primary myotubes are comparable to similar parameters measured in skeletal muscle tissue obtained from the same subjects

The primary aims for this study were therefore

- To determine the fiber type composition, intramyocellular lipid (IMCL) and mitochondria of primary myotubes obtained from athletes, sedentary lean and type 2 diabetic mellitus (T2DM) subjects, and
- To determine the effect of progressive passaging on fiber type composition, IMCL- and mitochondria contents in primary myotubes.
Study population, materials and methods

2 Study population, materials and methods

2.1 Materials

Dulbecco’s Modified Eagle Medium Low Glucose with L-glutamin (DMEM), Minimum Essential Media with Glutamax (MEM), fetal bovine serum (FBS), Fungizone (250µg/ml amphotericin B), Gentamicin (50mg/ml gentamicin sulfate), Human epidermal growth factor, Penicillin-streptomycin (10 000 IE / 10mg/ml), Dulbecco’s Phosphate Buffered Saline (DPBS), Hank Balance Salt Solution (HBSS), 10x Phosphate Buffered Saline (10xPBS), secondary antibody 680 mouse Alexafluor, Bodipy 493/503 and Mitotracker Green Probe were obtained from Invitrogen Molecular Probes (Carlsbad, CA, USA). Dexamethasone, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Formalin solution neutral buffered 10%, saponin, 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), Radio-Immunoprecipitation Assay (RIPA), 2-propanol 99.5+%, Corning® T-75 flasks, Corning® T-25 flasks, and cryovials were purchased from Sigma-Aldrich (St.Louis, MO, USA). Fetuin was obtained from Akron Biotech (Boca Raton, FL, USA). Trypsin neutralizing solution (TNS) and Trypsin/EDTA were obtained from Lonza (Houston, TX, USA). Bovine serum albumin was purchased from Jackson Immuno Research (Westgrove, PA, USA). Primary antibody myosin heavy chain I (MHC-I) mouse, Microcon® centrifugal filter devices were obtained from Millipore (Billerica, MA, USA). 12 well multiwall glass bottom culture plates were purchased from MatTek (Ashland, MA, USA). Mitochondria antibody mouse was purchased from Novus (Littleton, CO, USA). Pierce® BCA protein assay kit and 96 well Microlite 1+ flat bottom white plate were obtained from Thermo Scientific (Rockford, IL, USA). Tween 20, Criterion Precast 12% Tris-HCl 12 well gel, 10xTris/glycine/SDS (TGS) and 10xTris/glycine buffer (TG) were purchased from Bio-RAD (Hercules, CA, USA). 96 well Microplate flat bottom was purchased from Greiner Bio-one (Monroe, NC, USA). Odyssey blocking buffer was obtained from Li-Cor (Lincoln, NE, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Roche (Indiananapolis, IN, USA).
Study population, materials and methods

2.2 Study population

Experiments were conducted by using samples from ACTIV I, ACTIV II and Take time studies completed at PBRC. Subject characteristics are presented in table 2.1. Six subjects in each group were included in the experiment.

Table 2.1: Sample characteristics of athletes, lean and T2DM. Data are presented as mean ± standard deviation and analyzed using linear mixed model. Values with * in each row are significantly different from athletes and lean (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Athletes</th>
<th>Lean</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (M/F)</td>
<td>6 (6/0)</td>
<td>6 (6/0)</td>
<td>6 (4/2)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72.0±8.5</td>
<td>77.6±7.8</td>
<td>110.5±15.9 *</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.3±2.0</td>
<td>24.4±2.3</td>
<td>40.3±5.5 *</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.7±3.1</td>
<td>22.8±3.4</td>
<td>46.2±10.9 *</td>
</tr>
<tr>
<td>Race</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td></td>
</tr>
</tbody>
</table>

After signing the informed written consent approved by the PBRC ethical review board, patients were enrolled in one of two clinical trials performed in Baton Rouge, LA at the PBRC. Volunteers qualified and were enrolled in Clinicaltrials.gov NCT00402012 (TAKE TIME) if they had known T2DM were ‘diet-controlled’ or were taking metformin, insulin and/or sulfonylureas but not thiazolidinediones and were otherwise healthy.

Volunteers qualified and were enrolled in NCT00401791 (ACTIV) if they were age 20-40, had a body mass index (BMI) 20-30 kg/m², were non-diabetic, were taking no medications and were otherwise healthy. Subjects in this study were recruited based on their level of habitual physical activity and a family history of T2DM. Physical activity level was calculated from a 7-day physical activity questionnaire recall and a triaxial accelerometer worn for at least 4 days. Inclusion criterion for the sedentary and active groups was a VO2max lower than or above 40 ml/kg/min respectively. A family history of T2DM was considered positive if one first degree relative had T2DM.
2.3 Methods

2.3.1 Seeding muscle cells

Muscle cells were seeded and grown as described before [58]. In brief, sorted cells were thawed rapidly in a 37°C water bath. Thereafter, cells were seeded in four 75cm² flasks with 25ml of 10% DMEM growth media (appendix 7.1.1). Cells were distributed evenly by tipping the flasks back and forth. Cells were incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C.

2.3.2 Growing and differentiation of muscle cells into myotubes

Cells were grown with 10% DMEM media until it reached ~70% confluence. Media was changed every other day. When confluence was reached, two of the 75cm² flasks were frozen down into sixteen cryovials and seeded for next passage. This was performed by first washing the two 75cm² flasks with Hank Balance Salt Solution (HBSS) three times. 4ml of Trypsin 0.25% was added in each flask to detach cells. After cells were detached, 8ml of Trypsin Neutralizing Solution (TNS) was added to neutralize the Trypsin. Cells were then transferred to a 50ml conical tube and centrifuged at 25°C and 1170 rpm for 10 minutes. Supernatant was discarded and cell pellet was resuspended in 20ml 10% dimethyl sulfoxide (DMSO) solution (appendix 7.1.3), mixed thoroughly and seeded in new flasks ready for the next passage. Rest of the cells was aliquot in sixteen cryovials. Cryovials were frozen down in freezing container with propanol at -80°C for 24 hours, and thereafter transferred to liquid nitrogen tank.

The two remaining confluent 75cm² flasks were changed to 2% MEM media (appendix 7.1.4) in order to induce differentiation to myotubes. Cells were incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C and media was changed on alternate days. When cells reached confluence, cells were harvested for proteins (see section 2.3.5 – Protein harvesting) and RNA (see appendix 7.5 – Experiment: Real time Polymerase chain reaction - RNA).
2.3.3 Experiment: Immunohistochemistry – MHC-I quantification

Preparing the immunohistochemistry

As described earlier [62] with minor modifications, previously frozen down cells, respectively, passage 4, 5, 6, and 7, for each subjects, were plated on 12 well multiwall glass bottom culture plates (Cat#P12G014F, MatTek Corporation) with athletes, lean, and T2DM, attempting one subject from each group on the same plate as shown in figure 2.1. These were grown in duplicates. Frozen cells were thawed into 10% DMEM media in a conical tube and counted using traditional hemocytometor techniques. Cells were seeded into 12 well plates at a level of 76,000 cells per well, and were cultured in 10% DMEM media to ~70% confluence (3-5 days). After confluence, cells were differentiated with 2% MEM media for 5-7 days to ~70% confluence was reached.

Figure 2.1: Design of 12 well plates in immunohistochemistry experiment. Figure 2.1 shows the desired design of the immunohistochemistry experiment. Each plate had athletes, lean and T2DM donors and donors in each passage were grown in duplicates.

This experimental design was not accomplished because of the differences in growth rate among subjects, passage and cohorts e.g. cells from athletes had higher growth rate than cells obtained from T2DM. Subjects were therefore designed to grow by the cells growth rate (noted when growing in 75cm² flasks) on 12 well plates.

Immunostaining

Myotubes were stained as previously described [63]. Briefly, cells were fixed with 10% formalin solution at ~70% confluence after washing with HBSS. Cells were permeabilized using 0.1% saponin solution (appendix 7.2.1) and incubated for 20 minutes at room temperature on a rocker. Cells were rinsed with phosphate-buffered saline (PBS) (appendix 7.2.2). 3% bovine serum albumin (BSA) in PBS (appendix 7.2.3)
was added to the cells and incubated for 10 minutes at room temperature on a rocker. 200µl of the primary antibody MHC-1 mouse (cat#MAB1628, Millipore) 1:100 diluted solution (appendix 7.2.5) or mitochondria (cat#NB600.556, Novus) 1:100 diluted solution (appendix 7.2.6) was added as figure 2.2 describes. Plates were wrapped with parafilm and incubated for 1 hour at room temperature on a rocker. Cells were washed three times with 0.05% tween 20 in PBS (appendix 7.2.7). 200µl of secondary antibody Alexafluor (cat#21058, Invitrogen) 1:200 diluted solution (appendix 7.2.8) was added with lights off, and plates were covered with aluminum foil and incubated for 1 hour at room temperature on a rocker. Cells were washed three times with 0.05% tween 20 in PBS. 500µl of boron-dipyrromethene (bodipy) 493/503 (cat#D3922, Invitrogen Molecular Probes) 1:100 diluted solution (appendix 7.2.9) was added, and covered with aluminum foil and incubated at room temperature on a rocker. Cells were washed three to four times with PBS. 600µl of 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (cat#D8517, Sigma-Aldrich) 1:10000 diluted solution (appendix 7.2.10) was added and covered with aluminum foil and incubated for 10 minutes at room temperature on the bench. Cells were washed twice with PBS, and PBS was in the end added to the wells. Plates were wrapped with parafilm and covered with aluminum foil, and stored at 4°C.

Confocal imaging

Images were captured using Cell Biology and Bioimaging Core (CBBC) facility on the Leica SP5 confocal microscope (Leica, Bannockburn, USA) the day after staining was performed. Primary myotubes were stained for MHC-I, mitochondria, bodipy (lipid)
and DAPI (nucleus) and images were captured with a 20x0.70 IMM UV object (figure 2.3A). Two different channels were used, respectively acoustu-optical tunable filter (AOTF) 561 and AOTF 488, and the emission bandwidth was 515-545nm (PMT2) and 600-620nm (PMT3).

**Image analysis**

Images were analyzed using *MacBiophotonics Image J*. [64]. To determine fiber type-I in muscle cells, four pictures of each stained well were viewed simultaneously and converted to grey scale images (figure 2.3B). To asses the amount of fiber type-I, threshold was measured from stained images on a 0-100 000 intensity scale with 0 representing no visibly of stained fiber type-I and 100 000 representing maximum amount of fiber type-I (figure 2.3C). Pictures were also counted for the stained nucleus (DAPI) using the same software (figure 2.3D).

![Image analysis using MacBiophotonics Image J. A) MHC-I stained type-I fibers, B) MHC-I stained type-I fiber converted to Grey scale by MacBiophotonic Image J, C) Measurement of area covered with MHC-I, D) Counting DAPI for normalization.](image)

Figure 2.3: Image analysis using *MacBiophotonics Image J*. A) MHC-I stained type-I fibers, B) MHC-I stained type-I fiber converted to Grey scale by MacBiophotonic Image J, C) Measurement of area covered with MHC-I, D) Counting DAPI for normalization.
Study population, materials and methods

**Flexstation**

Additionally, Softamax PRO5 FLEX station (Molecular devices, USA) was used to quantify MHC-I, lipid content and nucleus, by measuring excitation at 365-464nm (DAPI), 485-538nm (bodipy), and 675-704nm (MHC-I). Wells were normalized for cell number obtained from DAPI staining.

MHC-I measured by this method was not usable because of inconsistent fluorescence signal from MHC-I. This resulted in difficulties averaging MHC-I. Also, certain areas in the culture wells showed signals that were consistently higher or lower in all the plates. These data were therefore not implicated in this study. Quantifying fiber type-I by immunohistochemistry technique gave reliable and accurate data, and was therefore preferred.

2.3.4 Experiment: Mitotracker – quantification of mitochondrial mass

Cryovials with cells from already frozen down passages (passage 4 to passage 7) were seeded in 25cm² flasks and grown. When ~70% confluence was reached, myoblasts were differentiated. Mitotracker was performed as described earlier [65]. Briefly, Mitotracker Green Probe (Cat#M7514, Invitrogen) solution (appendix 7.3.1) was added to flasks after washing with pre-warmed PBS two times. Flasks with Mitotracker were incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C for 30 minutes. Mitotracker solution was removed, and flasks were washed with PBS. 1ml of trypsin 0.25% was added to flasks and incubated until cells detached. Once cells detached, 2ml of TNS was added. The content in flasks was transferred to a 15ml conical tube and centrifuged at 900rpm for 5 minutes at 4°C. Supernatant was removed and pellet was resuspended in 2ml of PBS. Cells were counted using traditional hemocytometer techniques. 200µl of the resuspension was transferred to a 96 well Microlite 1+ flat bottom white plate (Cat#7571, Thermo). Fluorescence intensity was determined immediately on a Luminescence spectrometer model LS50B (PerkinElmer, East Lyme CT, USA) with excitation and emission wavelength of 490nm and 516nm.
2.3.5 Experiment: Western immunoblotting – protein quantification

Protein harvesting

0.25% trypsin and TNS were added the same way as for the freeze down (see section 2.3.2 – Growing and differentiation of muscle cells into myotubes) and centrifuged. 1.3ml protein buffer (appendix 7.4.1) was added to the cell pellet and mixed evenly. Cell suspension was transferred to an eppendorf vial, snap frozen in liquid nitrogen and stored at -80°C.

Protein concentration

Proteins were concentrated following protocol for Microcon® centrifugal filter devices from Millipore (Cat#UG-99394, Millipore). Briefly, proteins were centrifuged in a microcon centrifugal filter device until desired amount of liquid was left in the filter. This was transferred to a new eppendorf vial and protein concentration was measured by bicinchoninic acid (BCA) method.

Protein assay: Bicinchoninic assay (BCA)

5µl of concentrated cell suspension was transferred to a 96 well Microplate flat bottom as well as protein standards for BCA with known dilutions (5µg/µl, 2.5µg/µl, 1.25µg/µl, 0.625µg/µl, 0.3125µg/µl and 0.156µg/µl). 200µl protein dye in 1:50 dilution (appendix 7.4.2) was added to the wells with standards and cell suspension. The 96 wells plate was placed in an incubator at 37°C without CO₂ for 30 minutes. Protein concentration (µg/µl) was quantified with Spectramax model plus384 (Molecular devices, USA) at absorbance 562.

Western immunoblotting

Western immunoblotting was performed as described previously [66]. In brief, samples were prepared by mixing proteins with radioimmunoprecipitation assay (RIPA) buffer and dye in an eppendorf vial. This was vortexed and spun down, and heated to 95°C for 2 minutes. Samples were vortexed and spun down again, before loading to the Criterion Precast 12% Tris-HCl 12 well gel (cat#345-0009, BIO-RAD). The gel ran for 1 hour at
150 V and was transferred to a cassette for transferring the blot to the polyvinylidene fluoride (PVDF) membrane (cat#0301004001, Roche). The transferring ran for 40 minutes at 100V in cold room. After transferring was completed, the membrane was rinsed with milli-q water and blocking buffer was prepared (appendix 7.4.3). Blocking buffer was added to the membrane and blocked for 1 hour at room temperature on a rocker. The primary antibody MHC-I mouse (used for immunohistochemistry) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were made in a 1:1000 dilution and 1:1000 dilution (appendix 7.4.4). This was added to the membrane after discarding the blocking buffer. Primary antibody and GAPDH solution with membrane was incubated over night in cold room on a rocker. Next day, membrane was washed with tris-buffered saline tween 20 (TBS-T) (appendix 7.4.5) and TBS-T was thereafter added to the membrane and rocked at room temperature for 5-10 minutes. This step was performed three times. Secondary antibody 680 Alexafluor mouse was made (appendix 7.4.6) in a 1:7500 dilution and added to the membrane. This was incubated for 1 hour at room temperature on a rocker. Membrane was rinsed with TBS-T and TBS-T was added to membrane and rocked at room temperature for 5-10 minutes. This step was performed three times. Membrane was thereafter washed with tris-buffered saline (TBS) (appendix 7.4.7) two times for 5 minutes. Bands were visualized using Odyssey 9120 Infrared Imaging System (LI-COR, Lincoln, NE) and quantified using Odyssey Application Software version 2.1 (LI-COR, Lincoln, NE).

2.3.6 Clinical parameters

All clinical parameters used in this experiment were obtained from studies (ACTIV I, ACTIV II and Take time) conducted at PBRC. Physical parameters such as VO$_{2\text{max}}$, total daily energy expenditure (TDEE), metabolic equivalent task (MET) hours/week and physical activity level (PAL) were not measured in T2DM patients.

2.3.7 Statistical analysis

After the determination of fiber type-I, MHC-I protein, lipid content, and mitochondrial content, statistical analysis was performed to verify if any differences existed within the three different groups (athletes, sedentary and T2DM) using linear mixed model
Study population, materials and methods

(LMM) with Bonferroni correction as post-hoc analysis. LMM had the purpose to compare different passages within the same group, and to compare the difference between donors variation in the distinctive cohorts. Metabolic characteristics obtained from primary myotubes and recent experiments performed on skeletal muscle tissue were compared by linear regression analysis and by nonparametric Spearman's rho analysis. Clinical data were analyzed using LMM. Statistical analysis was performed using SPSS version 17 (SPSS, Chicago, IL) and GraphPad Prism version 5.0 (GraphPad Prism, La Jolla, CA). A P<0.05 was considered significant.
3 Results

3.1 SECTION 1: Baseline anthropomorphic measurements

Table 3.1: Subject characteristics conducted at PBRC. Data are presented as mean ± standard deviation and analyzed using linear mixed model (LMM) statistic. Values with * are significantly different from athletes. Values with ‡ are significantly different from lean. P<0.05 is considered significant. BMI, body mass index; VO₂max, maximal oxygen consumption; GDR, glucose disposal rate; EMBS, estimated metabolic body size; IMCL, intramyocellular lipid; ATP, adenosine 5’Triphosphate activated protein kinase.

<table>
<thead>
<tr>
<th></th>
<th>Athletes</th>
<th>Lean</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (M/F)</td>
<td>6 (6/0)</td>
<td>6 (6/0)</td>
<td>6 (4/2)</td>
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<tr>
<td>Weight (kg)</td>
<td>72.0±8.5</td>
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<td>110.5±15.9 *</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.3±2.0</td>
<td>24.4±2.3</td>
<td>40.3±5.5 *</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.7±3.1</td>
<td>22.8±3.4</td>
<td>46.2±10.9 *</td>
</tr>
<tr>
<td>VO₂max (ml/kg/min)</td>
<td>49.8±3.3</td>
<td>34.1±3.0 ‡</td>
<td>Not measured</td>
</tr>
<tr>
<td>GDR (mg/kg/EMBS/min)</td>
<td>12±2.2</td>
<td>7.6±2.0 ‡</td>
<td>3.3±1.6 *</td>
</tr>
<tr>
<td>Fiber type-I in VL (%)</td>
<td>50.0±20.0</td>
<td>40.0±20.0</td>
<td>40.0±20.0</td>
</tr>
<tr>
<td>IMCL in VL (AU)</td>
<td>15.4±8.1</td>
<td>5.5±4.7</td>
<td>9.9±9.1</td>
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<td>ATP_max (mM/sec)</td>
<td>1.1±0.1</td>
<td>0.7±0.1 ‡</td>
<td>0.5±0.1 ‡</td>
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<tr>
<td>ATPase (mM/sec)</td>
<td>7.2±1.7</td>
<td>5.3±0.9</td>
<td>3.1±1.4 ‡</td>
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</tbody>
</table>

Bajpeyi et al. collected clinical data conducted at PBRC and the characteristics of the study population are presented in table 3.1. Subjects in all groups were males except the T2DM group, which had both males and females. Diabetic subjects also had a significantly higher body weight and body mass index (BMI) compared to athletes and lean as expected. However, T2DM individuals were significantly older than athletes and lean subjects, which is one of the limitations of the present study. Moreover, VO₂max was significantly higher in athletes compared to lean, and glucose disposal rate (GDR) was significantly higher in athletes compared to lean and T2DM. There were no differences between the cohorts in fiber type-I composition and IMCL content measured in Vastus Lateralis using immunohistochemistry technique. However, using the same subjects, Bajpeyi et al. (unpublished) have recently observed a significant difference in fiber type-I and IMCL contents among athletes, lean and T2DM by increasing the subject
numbers [30]. Athletes had significant higher maximum mitochondrial capacity, measured by ATP$_{\text{max}}$, compared to lean and T2DM individuals, and significant higher resting rate of ATP synthesis, measured by ATPase, compared to lean and T2DM subjects.

3.2 SECTION 2: Differences in metabolic characteristics of primary myotubes obtained from athletes, lean and T2DM

The study was performed using primary myotubes cultured from six athlete, 6 lean and 6 T2DM donors at passage 4. The aim was to a) compare fiber type composition, lipid content and mitochondrial mass among three cohorts, b) to determine the effect of progressive passaging of cells on fiber type composition, lipid and mitochondrial mass (section 3.3) and c) to determine whether fiber type-I and lipid content measured in myotubes are comparable to the same parameters measured directly in skeletal muscle tissue obtained from the same donors (section 3.4 and 3.5).

Linear mixed model pairwise comparison with Bonferroni correction was performed as statistical analysis to identify whether there was any difference between the passages in fiber type-I, lipid content and mitochondrial mass measurements in the three groups. No significant difference was observed between the passages, thus all data from passage 4 to passage 7 were included in the following statistical analysis (n=48-54).

3.2.1 Difference in fiber type-I composition between myotubes established from athletes, lean and T2DM subjects

Fiber type-I characteristically has higher mitochondrial and IMCL content [45]. Bajpeyi et al. (unpublished) have recently shown higher percentage of type-I fibers measured in Vastus Lateralis in athletes compared to a sedentary lean and a T2DM population [30]. It was therefore interesting to determine if the same diversity was observed in cell culture model.
Figure 3.1: Fiber type-I measured in cell culture from athletes, lean and T2DM subjects. Human primary myotubes were established, grown and differentiated from passage 3 to passage 6 as previously described (section 2.3 Methods). Cells were reseeded into passage 4 to passage 7 at a level of 76.000 cells per well on 12 well plates, grown and differentiated. When ~70% confluence was reached, cells were fixed. Immunohistochemistry was performed day after fixing using primary antibody myosin heavy chain I mouse, mitochondria antibody mouse, DAPI and bodipy. Pictures were taken by Leica SP5 confocal microscope the day after staining and fiber type-I was analyzed using MacBiophotonics Image J. Data are presented as mean ± SEM and analyzed using linear mixed model. Bonferroni correction was applied for multiple comparisons. *Significantly lower percentage of fiber type-I than myotubes from athletes (9.9±1.8 vs. 1.6±1.0 vs. 2.0±0.8 AU; p<0.001).

Data illustrated that myotubes from athletes had a significant higher amount of fiber type-I compared to myotubes established from lean and T2DM. This resulted in an almost 80% higher content of fiber type-I in myotubes from athletes compared to lean and T2DM. No significant difference in fiber type was observed between myotubes from lean and T2DM groups (figure 3.1).
Results

**Figure 3.2:** Western immunoblot showing MHC-I protein in myotubes from athletes, lean and T2DM subjects. Human primary myotubes were established, grown and differentiated from passage 3 to passage 6 as previously described (section 2.3 Methods). At each passage, proteins were harvested and frozen down. Western immunoblotting was performed by using primary antibody myosin heavy chain I mouse and GAPDH. T2DM, Type 2 diabetes mellitus; MHC-I, myosin heavy chain I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P4, passage 4; P5, passage 5; P6, passage 6; P7, passage 7.

MHC-I protein expression was also examined in myotubes from athletes, lean and T2DM subjects by western immunoblotting. MHC-I protein was detected only in myotubes from athletes, whereas myotubes from lean and T2DM subjects there was no MHC-I protein detected (figure 3.2), again supporting the fiber type data using immunohistochemistry technique.

### 3.2.2 Difference in lipid content among myotubes from athletes, lean and T2DM subjects

It is essential to distinguish the lipid content in skeletal muscle since insulin resistance in T2DM occurs primarily due to an increase in lipid intermediates [17]. However, as already stated, increased lipid intermediates in endurance trained athletes are not associated with insulin resistance [16].

To quantify the difference in lipid content among myotubes from the three distinctive groups, immunohistochemistry with bodipy staining was performed and lipid content was measured by Flexstation and normalized to the cell density measured by DAPI staining (nuclei content). There was no significant difference in cell density among the groups at any passage, which confirmed that our data was not influenced by a disparity in cell density.
Results

Consistent with the literature where IMCL was measured in *Vastus Lateralis* muscle [23, 30], the lipid content measured in myotubes was significantly higher in athletes compared to myotubes from lean and T2DM subjects (figure 3.3). Cells from athletes had ~30% higher lipid content compared to lean and T2DM myotubes. There was no significant difference in lipid content between myotubes from lean and T2DM.

![Figure 3.3: Lipid content measured in cell culture from athletes, lean and T2DM.](image)

Human primary myotubes were established, grown and differentiated from passage 3 to passage 6 as previously described (section 2.3 Methods). Cells were reseeded into passage 4 to passage 7 at a level of 76,000 cells per well on 12 well plates, grown and differentiated. When ~70% confluence was reached, cells were fixed. Immunohistochemistry was performed day after fixing using primary antibody myosin heavy chain I mouse, mitochondria antibody mouse, DAPI and bodipy. Lipid content was quantified by Softmax PRO5 Flexstation at excitation wavelength 485-638nm. Data were normalized to nucleus (DAPI). Data are presented as mean ± SEM and analyzed using linear mixed model (LMM). Bonferroni correction was applied for multiple comparisons. *Significantly lower lipid content than cells from athletes (0.047±0.003 vs. 0.032±0.001 vs. 0.033±0.001 AU; p<0.001).

3.2.3 Difference in mitochondrial mass of myotubes from athletes, lean and T2DM subjects

Mitochondria are responsible for energy production by oxidative phosphorylation. Previous studies have demonstrated no significant difference in the mitochondrial mass in primary myotubes from lean and T2DM individuals [55, 65]. In this experiment we aimed to compare mitochondrial mass measured in myotubes obtained from athletes, lean and T2DM.
Results

Mitochondrial mass measured in primary cell culture was significantly higher in athletes compared to lean and T2DM. Furthermore, cells from athletes had ~20% more mitochondrial mass compared to myotubes from lean and T2DM subjects. No significant difference was observed between myotubes from lean and T2DM subjects (figure 3.4).

![Graph showing mitochondrial mass measured in myotubes from athletes, lean and T2DM.](image)

**Figure 3.4: Mitochondrial mass measured in myotubes from athletes, lean and T2DM.** Previously frozen cells were reseeded at passage 4 to passage 7 in 25cm² flasks, grown and differentiated as previously described (section 2.3 Methods). Mitotracker Green was added and cells were incubated for 30 minutes. Mitochondrial mass was determined by Luminescence spectrometer model LS508B at excitation and emission wavelength of 490nm and 516nm. Data are presented as mean ± SEM and analyzed using linear mixed model (LMM). Bonferroni correction was applied for multiple comparisons. *Significantly lower mitochondrial mass than myotubes from athletes (105.2±3.8 vs. 80.0±3.0 vs. 90.0±3.9 AU; p<0.01).

3.3 SECTION 3: Effect of increased passage number on fiber type composition, lipid content and mitochondrial mass of myotubes obtained from athletes, lean and T2DM subjects

This study was performed on myotubes obtained from athletes, lean and T2DM subjects grown from passage 4 to passage 7. The purpose of this experiment was to determine whether fiber type-I, lipid content and oxidative properties were retained throughout the subsequent passaging of the cells during growth and differentiation. Earlier studies have been performed on muscle cells from both rats [59] and pigs [60],
and the different markers (MyoD and IGFBP-2) confirmed a decrease or diminished differentiation capacity with progressive passaging. Nehlin et al. also proposed that human primary myotubes were altered when growing at multiple passages (passage 2, 5, 8 and 11). They observed that the proliferative potential, differentiation capacity and metabolic processes were decreased [54].

3.3.1 Effect of progressive passaging on fiber type-I

![Graphs showing changes in fiber type-I, lipid content, and mitochondrial mass with increasing passaging.]

Figure 3.5: Effect of increased passaging on A) fiber type-I measured in myotubes, B) lipid content measured in myotubes and C) mitochondrial mass measured in myotubes. Data are presented as mean ± SEM and analyzed using linear mixed model pairwise comparison. No significant differences were observed with the effect of passaging (P>0.05).

Fiber type-I was measured by immunohistochemistry technique in all three groups from passage 4 to passage 7. A decreasing trend in fiber type-I through passages in athletes, lean and T2DM myotubes was observed, as figure 3.5A illustrates. However, statistical analysis did not show any significant difference between the four passages.
Results

(passage 4 vs. passage 5 \( p > 0.90 \), passage 4 vs. passage 6 \( p > 0.35 \), passage 4 vs. passage 7 \( p > 0.14 \)).

3.3.2 Effect of progressive passaging on intracellular lipid content

Lipid content of myotubes was measured by Flexstation in all three donor groups from passage 4 to passage 7 after staining with bodipy. No significant difference with progressive passaging was observed as figure 3.5B illustrates (passage 4 vs. passage 5 \( p > 1.0 \), passage 4 vs. passage 6 \( p > 1.0 \), passage 4 vs. passage 7 \( p > 1.0 \)). However, performing correlation analysis in cell culture model with passage 4 vs. passage 5, passage 6 and passage 7, showed a correlation between lipid content in passage 4 vs. passage 5 and passage 4 vs. passage 6, but not in passage 4 vs. passage 7. This indicates that although the cellular lipid content in primary myotubes was retained with progressive passages (passage 5 and 6), this property was lost with successive increase in passage number (passage 7).

3.3.3 Effect of progressive passaging on mitochondrial mass

Mitochondrial mass was measured by Mitotracker Green in cells from all three groups from passage 4 to passage 7. No significant reduction in mitochondrial mass was observed with progressive passages (figure 3.5C). However, a slight trend of a decrease in mitochondrial mass was observed at passage 7 compared to passage 4 (Passage 4 vs. passage 5 \( p > 1.0 \), passage 4 vs. passage 6 \( p > 0.5 \), passage 4 vs. passage 7 \( p = 0.05 \)).

3.4 SECTION 4: Relationships between fiber type-I measured \textit{in vitro} and \textit{in vivo} parameters

The purpose of these calculations was to investigate whether metabolic components such as fiber type-I content measured in primary myotubes was comparable to the same measured in skeletal muscle tissue obtained from the same subjects. Data collected from myotubes at passage 4 from the 18 subjects (six athletes, 6 lean and 6 T2DM) were used for this analysis. Additional correlation analysis was studied in all
Results

the four passages of fiber type-I as no differences were observed between distinctive passages (section 3.2 and 3.3 - Results).

3.4.1 Fiber type-I content measured in cell culture is associated with fiber type-I content measured in tissue.

A)  

B)  

Figure 3.6: Fiber type-I shown in A) cell culture and in B) tissue. Figure 3.6 A) shows staining of fiber type-I in primary myotubes. Red represents fiber type-I, turquoise represents nucleus and green represents lipids. Figure 3.6 B) shows fiber type-I in frozen skeletal muscle. Red represents fiber type-I, blue represents fiber type-II, and green represent intramyocellular lipid (IMCL).

Bajpeyi et al. (unpublished) have successfully stained type-I fibers in human skeletal muscle tissue (figure 3.6 B) [30]. Red and blue colors indicate type-I and type-II fibers respectively. Using the same antibody (mouse anti-slow muscle myosin monoclonal antibody) we successfully stained fiber type-I myotubes in human primary cell culture (figure 3.6 A). Red represents fiber type-I, the turquoise area represents the nucleus, and green represents lipid content in human primary myotubes. Correlation analysis between fiber type-I measured in cell culture and fiber type-I measured in tissue (Vastus Lateralis) was performed to study whether our cell culture model of primary myotubes preserved similar fiber type characteristics as in skeletal muscle tissue.
Results

Figure 3.7: Correlation between fiber type-I measured in cell culture and fiber type-I measured in tissue (Vastus Lateralis). Fiber type-I data from tissue were taken from ACTIV I, ACTIV II and Take time studies conducted at PBRC. Fiber type-I data from cell culture (passage 4) was measured by immunohistochemistry using mouse anti-slow muscle myosin monoclonal antibody. Data were analyzed using linear regression analysis. Subjects that had no (zero) fiber type-I expressed in cell culture were not included in this correlation.

Figure 3.7 illustrates a significant and positive relationship between fiber type-I measured in cell culture and fiber type-I measured in tissue (Vastus Lateralis) ($r^2=0.42$, $p<0.042$). It should be noted that the analysis was performed using only the subjects that expressed fiber type-I in primary myotubes ($n=10$), thus not including subjects that expressed zero MHC-I (no MHC-I). Consistent with the data already collected (figure 3.1) and previous studies in muscle tissue (Bajpeyi et al. unpublished data) [30], athletes had both higher amount of fiber type-I in cell culture and in tissue compared to lean and T2DM donors. To verify these results, an additional nonparametric Spearman’s correlation analysis was performed including fiber type-I data from passage 4 to passage 7 versus fiber type-I measured in tissue. Fiber type-I measured in vivo was positive associated with fiber type-I at all passages (Spearman’s $r=0.34$, $p<0.006$), thus consistent with correlation analysis performed in fiber type-I measured in myotubes (passage 4) versus fiber type-I measured in tissue.

3.4.2 Fiber type-I measured in cell culture is associated with mitochondrial function measured in vivo

Maximal ATP synthesis rate ($ATP_{\text{max}}$) measures the functional property of the mitochondria in vivo, which reflects the mitochondrial content and the mitochondrial
efficiency [67]. ATPase reflects the mitochondrial ATP flux in resting skeletal muscle [28].

Figure 3.8: Correlation between fiber type-I measured in cell culture and ATP$_{\text{max}}$ and ATPase measured in tissue (Vastus Lateralis). ATP$_{\text{max}}$ and ATPase were taken from ACTIV I, ACTIV II and Take Time studies conducted at PBRC. Fiber type-I data from cell culture (passage 4) was measured by immunohistochemistry using mouse anti-slow muscle myosin monoclonal antibody. Data were analyzed using linear regression analysis.

ATP$_{\text{max}}$ and ATPase measurements in Vastus Lateralis were collected in previous study performed by Bajpeyi et al. [28]. Figure 3.8A illustrates a positive and significant relationship between ATP$_{\text{max}}$ and fiber type-I measured in cell culture ($r^2=0.42$, $p<0.026$). Athletes had higher content of fiber type-I in cell culture (figure 3.1), which also reflected a high ATP$_{\text{max}}$ activity in tissue compared to lean and T2DM subjects. Similar to ATP$_{\text{max}}$, figure 3.8B shows a positive and significant correlation between ATPase and fiber type-I measured in myotubes ($r^2=0.34$, $p<0.047$). The correlation indicated that athletes had higher resting ATP flux compared to lean and T2DM subjects. Correlation analysis performed on all four cell passages on fiber type-I content (passage 4 to passage 7) versus ATP$_{\text{max}}$ and ATPase measured in tissue, verified these results (ATP$_{\text{max}}$: Spearman’s $r=0.53$, $p<0.001$; ATPase: Spearman’s $r=0.53$, $p<0.001$).

3.5 SECTION 5: Relationships between lipid content in vitro and in vivo parameters

The purpose of these calculations was to determine whether metabolic components such as lipid content in primary myotubes was comparable to the same measured in
skeletal muscle tissue obtained from the same subjects. Additional correlation analysis was performed in all the four passages of lipid content since no differences were observed between distinctive passages (section 3.2 and 3.3 - Results).

3.5.1 Lipid content measured in cell culture is not associated with IMCL measured in muscle tissue (*Vastus Lateralis*)

![Correlation between lipid content measured in cell culture and lipid content measured in tissue (*Vastus Lateralis*)](image)

*Figure 3.9: Correlation between lipid content measured in cell culture and lipid content measured in tissue (*Vastus Lateralis*). IMCL content in muscle tissue was obtained from the ACTIV I, ACTIV II and Take Time studies conducted at PBRC. Lipid content was measured in cell culture (passage 4) by immunohistochemistry using bodipy. Data were analyzed using linear regression analysis. IMCL content in tissue was measured by immunohistochemistry technique by Bajpeyi et al. (unpublished) [30]. Figure 3.9 showed no significant association between lipid content from *in vivo* (*Vastus Lateralis*) and lipid content from *in vitro* experiments ($r^2=0.001$, $P>0.9$), which was contrary to our aims. Correlation analysis studied in all four cell passages (passage 4 to passage 7) versus IMCL measured in tissue, confirmed this disassociation (Spearman’s $r=0.199$, $P<0.14$).*
3.5.2 Lipid content measured in cell culture versus fiber type-I properties from muscle tissue (*Vastus Lateralis*)

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 3.10**: Correlations between lipid content measured in cell culture versus A) fiber type-I, B) fiber size of type-I fiber and C) fiber density of fiber type-I obtained from *Vastus Lateralis*. Fiber type-I, fiber size, and fiber density data were taken from ACTIV I, ACTIV II and Take Time studies conducted at PBRC. Lipid content was measured in cell culture (passage 4) by immunohistochemistry using bodipy. Data were analyzed using linear regression analysis.

We have already shown that the lipid content measured in myotubes was higher in athletes than in lean and T2DM subjects (figure 3.3). Bajpeyi et al. (unpublished) have shown that athletes have higher amount of fiber type-I compared to lean and T2DM measured *in vivo* [30]. Performing correlation analysis between lipid content measured in primary myotubes and type-I fiber in *Vastus Lateralis* gave a highly significant positive correlation ($r^2=0.47$, $p<0.003$) (figure 3.10 A). This correlation demonstrated that athletes had a higher lipid content in myotubes, which reflected the higher amount of fiber type-I observed in tissue. Nevertheless, this association is consistent with data obtained from tissue [30] and fiber type-I measured in cell culture (figure 3.1).
Results

Correlation analysis was performed on all the four cell passages versus fiber type-I in tissue. Similar to previous correlation, a highly significant relationship was observed between lipid content in myotubes at all four passages versus fiber type-I measured in tissue (Spearman’s \(r=0.43\), \(p<0.001\)).

A balance between myofibrillar protein synthesis, mitochondrial biosynthesis and degradation determine the fiber size in skeletal muscle. Recent studies have shown that fibers with high oxidative capacity are relatively small [68]. Figure 3.10B demonstrates a positive and significant relationship between lipid content and fiber size in *Vastus Lateralis* muscle \((r^2=0.261, P<0.043)\). Correlation indicated that athletes tended to have larger fiber size with a relatively high lipid content in cell culture, while lean and T2DM tended to have smaller fiber size with lower amounts of lipid in cell culture. However, a positive trend \((p>0.06)\) but not significant was observed when correlation of lipid content measured in cell culture was studied at all the four cell passages versus fiber size measured in *Vastus Lateralis* (Spearman \(r=0.25\), \(p>0.06\)).

Fiber density is a measure of fiber type distribution that considers the volume of the fiber where both size and area covered with fiber type-I are included in the assessment [30]. Figure 3.10C illustrates a positive and significant relationship between lipid content in cell culture and the fiber type-I density in *Vastus Lateralis* \((r^2=0.44\), \(p<0.005\)). Moreover, athletes had higher lipid content in cell culture, which reflected higher fiber density compared to lean and T2DM subjects. Looking into the whole number of lipid data from passage 4 to passage 7 versus fiber type-I density measured in tissue, a similar relationship was observed (Spearman’s \(r=0.47\), \(p<0.001\)).
3.5.3 Lipid content measured in cell culture versus mitochondrial properties measured in tissue

![Figure 3.11: Correlations between lipid content measured in cell culture and ATP<sub>max</sub> and ATPase measured in tissue (Vastus Lateralis). ATP<sub>max</sub> and ATPase data were taken from ACTIV I, ACTIV II and Take time studies conducted at PBRC. Lipid content was measured in cell culture (passage 4) by immunohistochemistry using bodipy. Data were analyzed using linear regression analysis.]

As stated earlier, ATP<sub>max</sub> and ATPase data were taken from a previous study performed by Bajpeyi et al. [28]. As presented in figure 3.11A, a positive and highly significant relationship between lipid content measured in cell culture and ATP<sub>max</sub> measured in vivo (r²=0.58, p<0.001) was observed. Athletes had higher lipid content, which indicated higher ATP<sub>max</sub>, whereas lean and T2DM had lower lipid content, thus indicating lower ATP<sub>max</sub>. In figure 3.11B, no evidence of a relationship between lipid content measured in cell culture and ATPase measured in vivo was observed (r²=0.14, p>0.17). Performing correlation with all four lipid passages versus ATP<sub>max</sub> (Spearman’s r=0.60, p<0.001), strengthen the association observed between lipid content measured in cell culture (passage 4) versus ATP<sub>max</sub>. Contrary to above correlation (lipid content passage 4 vs. ATPase), lipid content in all four passages versus ATPase, showed a significant relationship (Spearman’s r=0.4, p<0.01). This could be due to the increased n when all four passages were included (n=56 vs. n=14).
Results

3.5.4 Lipid content measured in cell culture versus physical fitness and physical activity level measured by VO$_{2\text{max}}$, MET, TDEE and PAL in a non-diabetic population

![Graphs showing correlations](image)

Figure 3.12: Correlations between lipid content measured in cell culture and VO$_{2\text{max}}$, MET hours per week, TDEE and PAL measured in non-diabetic subjects. VO$_{2\text{max}}$, MET hours per week, TDEE and PAL data were taken from the ACTIV I and ACTIV II studies conducted at PBRC. Lipid content was measured in cell culture (passage 4) by immunohistochemistry using bodipy. Data were analyzed using linear regression analysis. VO$_{2\text{max}}$, maximal oxygen consumption; MET, metabolic equivalent task; TDEE, total daily energy expenditure; PAL, physical activity level; RMR, resting metabolic rate.

Previous study has already shown that VO$_{2\text{max}}$ in athletes is higher compared to lean [28]. Lipid content measured in myotubes of athletes was also significantly higher than lean as presented earlier (figure 3.3). Comparing physical fitness measured by VO$_{2\text{max}}$ and lipid content measured in cell culture, confirmed that higher VO$_{2\text{max}}$ in human subjects was correlated with higher content of lipid in cell culture in a non-diabetic population ($r^2=0.54$, $p<0.024$) (figure 3.12A). This observation was also seen when lipid content from all four passages versus VO$_{2\text{max}}$ was studied (Spearman’s $r=0.43$, $p<0.008$).
Results

Physical activity is measured by metabolic equivalent (MET) hours per week, reflecting both exercise intensity and amount of exercise per week. As figure 3.12B presents, a positive and highly significant relationship is observed between lipid content measured in cell culture and MET hours/week measured in a non-diabetic population ($r^2=0.81$, $p<0.001$). Athletes had higher lipid content, reflecting higher MET hours/week in donors compared to lean subjects. The same positive and highly significant observation was illustrated when lipid content in myotubes from all four passages versus MET hours/week was studied (Spearman’s $r=0.64$, $p<0.001$).

Similar to VO$_{2\text{max}}$ and MET hours/week, a positive and significant relationship was observed between lipid content measured in cell culture and total daily energy expenditure (TDEE) in a non-diabetic population ($r^2=0.75$, $p<0.001$) (figure 3.12C). Furthermore, athletes had higher lipid content, which indicated higher TDEE compared to the lean population, as we would expect. Looking into the lipid content in myotubes at all four passages versus TDEE, showed a consistent relationship (Spearman’s $r=0.54$, $p<0.001$) as observed for lipid content in myotubes from passage 4 versus TDEE.

Physical activity level (PAL) is a measurement, expressed as TDEE/resting metabolic rate (RMR). Comparable with previous physical activity parameters, a highly significant association was observed between lipid content measured in myotubes and PAL ($r^2=0.85$, $p<0.0001$) measured in human subjects (figure 3.12D).

Overall, our results showed that high lipid content measured in cell culture reflected a better physical fitness (VO$_{2\text{max}}$) and higher physical activity level (MET and PAL).
Results

3.5.5 Lipid content measured in cell culture is associated with insulin sensitivity measured by hyperinsulinemic euglycemic clamp.

Figure 3.13: Correlation between lipid content measured in cell culture and GDR measured in human subjects. GDR data was obtained from the ACTIV I, ACTIV II and Take time studies conducted at PBRC. Lipid content was measured in cell culture (passage 4) by immunohistochemistry using bodipy. Data were analyzed using linear regression analysis. GDR, glucose disposal rate; EMBS, estimated metabolic body size.

Recent study demonstrated a high glucose disposal rate (GDR) in athletes, and a low GDR in T2DM subjects [28]. Looking into the relationship between GDR and lipid content in cell culture (figure 3.13), a positive and significant association was observed ($r^2=0.32$, $p<0.021$). Subjects with high lipid content (athletes) reflected a better insulin sensitivity in vivo compared to lean and T2DM subjects. This finding is consistent with current literature [28]. The same positive and highly significant correlation was observed when lipid content in myotubes from all four passages versus GDR was studied (Spearman’s $r=0.35$, $p<0.007$).
Table 3.2: Summary of correlation analysis performed on fiber type-I and lipid content in primary myotubes vs. in vivo parameters. Fiber type-I and lipid content measured in myotubes were correlated to distinctive in vivo parameters measured in Vastus Lateralis samples. Correlation analysis of passage 4 (fiber type-I and lipid measured in myotubes) was performed using linear regression model (GraphPad version 5.0). Nonparametric Spearman’s analysis (SPSS version 17) was used to study correlation data from myotubes at all passages (passage 4 to passage 7). P<0.05 was considered significant. VL, Vastus Lateralis; IMCL, intramyocellular lipid; ATP, adenosine triphosphate; MET, metabolic equivalent task; TDEE, total daily energy expenditure; PAL, physical activity level; GDR, glucose disposal rate; EMBS, estimated metabolic body size.

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<th>r²</th>
<th>P-value</th>
<th>Spearman’s r</th>
<th>P-value</th>
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<td></td>
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<td>• Fiber type-I in VL (%)</td>
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<td>• IMCL in VL (AU)</td>
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<td>• ATPase (mM/sec)</td>
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<td>• IMCL in VL (AU)</td>
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<td>P&gt;0.90</td>
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<td>• Fiber type-I in VL (%)</td>
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<td>P&lt;0.003</td>
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<td>• Fiber size of fiber type-I in VL (AU)</td>
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<td>• ATPase (mM/sec)</td>
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<td>• VO&lt;sub&gt;2max&lt;/sub&gt; (ml/kg/min)</td>
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<td>P&lt;0.02</td>
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<td>• MET hrs/week</td>
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</tr>
<tr>
<td>• GDR (mg/kgEMBS/min)</td>
<td>0.3</td>
<td>P&lt;0.02</td>
<td>0.35</td>
<td>P&lt;0.007</td>
</tr>
</tbody>
</table>
4 Discussion

In this study, fiber type-I was identified in human primary myotubes. To our knowledge, type-I fiber has never been distinguished by immunohistochemistry in cell culture. Moreover, fiber type-I and IMCL content measured in myotubes were also comparable to clinical measurements performed independently in tissue, implying the important metabolic characteristics of this cell culture model. IMCL measured in myotubes did not correlate with IMCL measured in tissue (Vastus Lateralis). However, IMCL measured in myotubes was found to associate with clinical phenotypes like VO$_{2 \text{max}}$, activity level (MET and PAL) and GDR. Measuring fiber type-I, IMCL and mitochondrial mass with the effect of increased passaging in myotubes, a decreasing trend was observed for fiber type-I and mitochondrial mass. IMCL content was unchanged with increased passaging.

4.1 Role of metabolic characteristics in distinctive cohorts on fiber type-I, IMCL and mitochondrial mass

4.1.1 Fiber type composition

Cell culture model obtained from muscle tissue is of great importance and benefit due to the advantage of manipulating the biological system and mirroring clinical metabolic properties of subjects [62]. Additionally, the cell environment can be controlled precisely, which allows us to study the genetic, as well as epigenetic characteristics of the donors. Metabolic conditions in vivo can therefore be manipulated to understand critical metabolic pathways, functions and various physiological responses to drugs, substrates and metabolic stimuli on a genetic level [56, 61]. As fiber type composition plays a critical role in skeletal muscle metabolism, it is important to evaluate if primary myotubes actually retain the fiber type characteristics of skeletal muscle in vivo. It was therefore critical to study the difference on metabolic characteristics (fiber type-I, lipid and mitochondrial mass) of primary myotubes from athletes, lean and T2DM subjects, and whether these primary myotubes were comparable to metabolic parameters measured directly in muscle tissue.
Fiber type composition plays a significant role in determination of skeletal muscle metabolic characteristics. Several studies that determined fiber type composition in *Vastus Lateralis* muscle have demonstrated higher content of fiber type-I in endurance trained athletes compared to healthy lean subjects, higher content of fiber type-I in lean population compared to obese subjects, and lower fiber type-I content in T2DM compared to lean and obese controls [34, 40, 46, 48, 50, 51]. Moreover, Bajpeyi et al. (unpublished) reported higher percentage of type-I fibers in athletes compared to a sedentary lean and a T2DM population [30]. Our study demonstrated the same pattern in fiber type-I as current literature i.e., higher amount of type-I fiber in myotubes obtained from athletes, and lower amount of type-I fiber in lean and T2DM myotubes (figure 3.1). In addition, fiber type-I measured in myotubes was significantly associated with fiber type-I measured in frozen *Vastus Lateralis* muscle tissue (figure 3.7). Lipid content in myotubes was also found to positively associate with fiber type-I measured in *Vastus Lateralis* (figure 3.10A). Apart from measuring type-I fiber using immunohistochemistry, we also measured MHC-I in primary myotubes using Western immunoblotting. MHC-I protein was only expressed in myotubes obtained from athletes whereas myotubes from lean and T2DM subjects expressed little or no MHC-I (figure 3.2). These findings are important as it supports the idea that athletes have both higher fiber type-I content in myotubes, and that fibertype phenotype is preserved in cell culture model compared to when it is measured *in vivo*.

However, whether these differences in fiber type-I composition is due to the actually phenotype variation among the cohorts and/or because of the difference in differentiation capacity between the groups in myotubes (i.e. MHC-I expression is dependent on the capacity myoblasts have to fuse with each other forming myotubes), remains unclear. It can further be speculated whether MHC-I expression in these myotubes is dependent on an adequate differentiation process (capacity of myoblasts to fuse), or that myotubes ability to express MHC-I is dependent on the characteristics/phenotype of the donor i.e. T2DM and sedentary lean donors express less MHC-I. Remels et al. [69] reported that myoblasts from mice, contained extremely low levels of MHC-I, whereas myoblasts that had been differentiated into myotubes for 5-7 days contained more MHC-I compared to undifferentiated myoblasts. In our
experiment, myotubes were differentiated for 5 days or more (depending on the cohort) before quantifying MHC-I. This illustrates that a complete differentiation process is important for the expression of MHC-I. Moreover, Gaster et al. reported that expression of MHC-I in human primary myotubes might be blocked by missing factors since only a few myotubes expressed MHC-I when differentiated for 8 days, whereas MHC-II was expressed in all myotubes. These missing factors could be innervation or activity, which in vivo studies suggest are essential factors to retain the full slow fiber phenotype [56]. Additionally, Remel et al. and Gaster et al. performed the experiment on healthy mice and healthy donors [56, 69]. These experiments might not reflect athletes, lean and T2DM population we are studying because of a possible difference in the differentiation capacity between cohorts. In an ongoing study at PBRC (Galgani et al.), they found a lower MHC content in T2DM subjects compared to matched non-diabetic controls, which may indicate an impaired differentiation capacity of myotubes from these subjects. This point towards that T2DM muscle cells might need more than 5 days to fully express fiber type-I, which is in accordance with the differentiation process performed in our experiment. Although these factors are critical to sustain a full slow fiber phenotype, culturing human primary myotubes has the advantage that the environment can be precisely controlled, which allows us to study the genetic characteristics of the donors [55].

One of the limitations of our study is that T2DM subjects were markedly older than athletes and lean subjects. However, Proctor et al. have shown that fiber type-I is not affected by age [70], thus, higher content of fiber type-I in athletes compared to lean and T2DM, may not be influenced by this confounding factor.

4.1.2 Lipid content

Type-I muscle fibers have shown to be more oxidative compared to type II fibers [34, 46]. Endurance trained athletes contain more IMCL in type-I fibers and are highly insulin sensitive compared to the T2DM population. This refers to “The athlete paradox” which illustrates the association between insulin sensitivity and IMCL content in skeletal muscle [23]. Moreover, Moro et al. showed that this relationship was rather U-shaped than linear, proposing a high IMCL content and low insulin sensitivity in
Discussion

T2DM, and a high IMCL and high insulin sensitivity in athletes [18]. Environmental factors such as diet and activity level play important roles in determining the IMCL content of skeletal muscle tissue. We measured IMCL content in myotubes, which has a substantial advantage as it avoids environmental influence on this metabolic parameter. Our results demonstrate a higher IMCL content in primary myotubes obtained from athletes (figure 3.3). Furthermore, lipid content measured in myotubes was found to correlate with size of type-I fiber, fiber type-I content, and the fiber density of fiber type-I (figure 3.10). Thus, high IMCL content in myotubes obtained from athletes, indicate that the oxidative fibers are bigger in size and denser. A denser fiber reflects more mitochondria and higher lipid content in type-I fibers. These results are in agreement with current literature on muscle tissue, which suggest that muscle fiber type-I contains more IMCL and are more oxidative compared to type-II fibers [23, 46, 71]. This supports our hypothesis regarding translation of metabolic characteristics from in vivo back to in vitro, thereby preserving fiber type characteristics.

Despite the fact that type-I fibers are highly oxidative and have a high capacity for protein synthesis (myofibrillar proteins and proteins associated with oxidative metabolism), these fibers are relatively smaller in size compared to type-II fibers [68]. As figure 3.10B shows, athletes have high lipid content but tend to vary in fiber size, but having relatively large fiber size compared to lean and T2DM donors. This could be explained by the high lipid content in athletes, which may favor the synthesis of proteins associated with oxidative metabolism (increased mitochondrial biogenesis) rather than synthesis of myofibrillar proteins (increased muscle size) thus increasing the lipid turnover in skeletal muscle. However, several other mechanisms are responsible for the relationship between fiber type and oxidative metabolism. Further investigation is therefore needed to clarify this issue.

It has also been hypothesized that dysregulation of IMCL metabolism (i.e. elevated IMCL and/or lipid intermediates) in T2DM subjects are responsible for development of insulin resistance in skeletal muscle in vivo [18, 27, 72]. However, Goodpaster et al. reported an increased IMCL in endurance-trained subjects with high insulin sensitivity.
This finding suggests that an elevated IMCL content itself is not causally related to insulin resistance [23]. As stated earlier, our results performed in primary myotubes are in agreement with the current literature that athletes have higher lipid content measured in skeletal muscle compared to lean and T2DM subjects. Lipid content measured in cell culture was significantly correlated with GDR measured by hyperinsulinemic euglycemic clamp (figure 3.13). Moro et al. claimed that the IMCL turnover is dependent on the co-localization of intramyocellular lipid droplets and mitochondria for a complete mitochondrial β-oxidation [18]. Athletes had significant higher lipid content in myotubes compared to myotubes from lean and T2DM individuals. Nevertheless, studies in tissue show that IMCL turnover in athletes is much higher, which may determine the increased insulin sensitivity in athletes compared to T2DM. T2DM tends to generate lipotoxic metabolites because of reduced oxidative capacity and disparity of co-localization of IMCL droplets and mitochondria [18, 22]. Furthermore, no significant difference in lipid content in cell culture was observed between lean and (obese) T2DM myotubes. This suggests that lipid content might not play as an important role in the genetic etiology of T2DM, like previous study also indicates [73]. A known drawback is the age of the T2DM donors as already indicated. It can thus be speculated whether the insulin resistance in T2DM subjects is a cause of the age (insulin resistance develops with aging [74]) and/or is a direct consequence of the disease. However, Amati et al. suggested that insulin resistance was not associated with age, but rather associated with obesity and physical inactivity [21].

Correlation analysis between lipid content measured in cell culture versus IMCL measured in tissue (Vastus Lateralis) showed no relationship (figure 3.9). As stated earlier, cell culture model has the advantage of avoiding environmental factors, focusing on the genetic/epigenetic characteristics. Thus, lipid content measured in myotubes may be a genetic characteristic while lipid content measured in tissue is affected by environmental factors such as diet and level of physical activity. This might be a critical factor why lipid content measured in myotubes and IMCL measured in tissue did not correlate. However, a high fat diet can be mimicked in cell culture model by addition of fatty acids in the growth and differentiation media. Thus, adding lipid to the cells in future experiments may show an association between lipid content
measured in myotubes vs. IMCL measured in tissue. In our cell culture model, there was no apparent difference in distribution pattern of lipid in the myotubes, and lipid distribution also did not seem to be different based on fiber type of the myotubes (detected by microscope and by looking at stained fiber type-I and lipid images). The total lipid content in the area was therefore included when measuring. However, in tissue only the lipid inside the muscle cells were measured (excluding cell membrane and extramyocellular lipid). Images of lipid staining in cell culture did not illustrate any differences in distribution among type-I and type-II fibers (figure 3.6). Also, it was difficult to evaluate if the lipid was extramyocellular or intramyocellular lipid because of the distribution patterns of myotubes (density of the myotubes and growth of myotubes in more than one plane), which might have played a role in the disassociation between lipid content measured in myotubes and IMCL in skeletal muscle tissue. However, measuring lipid content in myotubes is of great importance as this is associated with several metabolic parameters measured independently in clinical settings such as VO$_{2\text{max}}$, PAL, and GDR.

Physical training (aerobic) is a significant element, which is associated with insulin sensitivity, VO$_{2\text{max}}$ and mitochondrial respiration [75]. In athletes, our results illustrated higher lipid content measured in cell culture compared to the lean population (figure 3.3). This finding is also described in tissue [23, 30]. Examination of lipid content measured in cell culture versus physical fitness and physical activity parameters (VO$_{2\text{max}}$, MET hours/week, TDEE and PAL) in a non-diabetic population, showed a strong relationship (figure 3.12.). Thus, athletes that have a high lipid content in myotubes, preserve the donors phenotype of physical fitness and physical activity characteristics. Several studies support this finding by illustrating that IMCL is an important source of energy for skeletal muscle during endurance exercising [76, 77]. We therefore propose that a high lipid content measured in myotubes reflects a better physical fitness of the donor. To our knowledge, this novel finding has never been shown before in cell culture and is therefore of great importance as it demonstrates that myotubes preserve the distinguished phenotype of a donor such as physical fitness and insulin sensitivity.
4.1.3 Mitochondrial mass

Several studies have recently demonstrated no difference in mitochondrial mass between healthy lean and obese T2DM subjects [55, 65]. Moreover, endurance trained athletes show higher mitochondrial content and oxidative capacity compared to a sedentary population [70, 78]. In our experiment, mitochondrial mass was higher in myotubes from athletes compared to myotubes established from lean and T2DM donors (figure 3.4). This suggests that myotubes from athletes have higher oxidative capacity compared to myotubes obtained from lean and T2DM individuals, with the assumption that mitochondria from all groups have similar characteristics. Corpeleijn et al. also reported that the rate of IMCL oxidation was dependent on mitochondrial function rather than mitochondrial mass [55].

Correlation analysis between fiber type-I measured in cell culture versus ATP$_{\text{max}}$ measured in the same donors, showed a positive association (figure 3.8) thus strengthen our finding regarding higher oxidative capacity in athletes. This finding was also observed in tissue where fiber type-I was found to correlate with ATP$_{\text{max}}$ [28]. Our results also showed that lipid content measured in myotubes was positively associated with ATP$_{\text{max}}$ measured in the same donors (figure 3.11). This additionally supports our findings regarding athletes having higher amount of fiber type-I, lipid content and mitochondrial mass (indicating that the oxidative capacity is higher), which reflects the in vivo phenotype of the donors. However, with increasing age, the ATP demand is found to be decreased [79]. Our T2DM subjects were older than athletes and lean subjects, thus this could possibly be a significant factor in the decreasing demand for ATP in T2DM donors.

Additionally, Kelley et al. reported smaller mitochondria in obese T2DM subjects and bigger mitochondria in a lean control population. It was also stated that age is one known factor that affects the size of mitochondria [47]. As mentioned above, T2DM donors were older than athletes and lean subjects. Although no statement concerning size can be made, it would be critical to study if these T2DM myotubes have smaller mitochondria compared to cells from lean and athletes, as we would expect from current literature and our findings in this study.
Discussion

Looking into type-I fiber, lipid content and mitochondrial mass measured in myotubes, the relative expression of fiber type-I, lipid content and mitochondrial mass when compared among athletes, lean and T2DM was similar in both the cell culture model and in tissue. Moreover, one of our main finding was that lipid content measured in myotubes did not correlate with IMCL measured in tissue. However, lipid content measured in myotubes correlated with clinical characteristics such as fiber type-I, ATP$_{\text{max}}$, VO$_{\text{2max}}$, PAL and GDR. These findings propose that the characteristics of the in vivo phenotype can be translated to myotubes in vitro by genetic or epigenetic mechanisms.

4.2 The effect of progressive passaging on metabolic characteristics of primary myotubes

Earlier studies have been completed on muscle cells obtained from rat and pig skeletal muscle, and demonstrated a decrease in MyoD and IGFBP-2, and a diminished proliferation capacity with progressive passaging [59, 60]. Moreover, Nehlin et al. described a reduced lipid metabolism in human primary myotubes and an impaired fusion degree of myoblasts with increasing passages (passages 2, 5, 8 and 11) [54]. However, it is not known whether there is a loss of fiber type-I due to the passage effect as there are no environmental factors such as physical activity during culturing of myotubes. This may be critical since cells are cultured through different passages and used for subsequent experiments without knowing if the heterogeneity in cell culture is retained. Our hypothesis proposed a decreased content of fiber type-I, lipid content and mitochondrial mass with increasing passaging.

The results described a decreased tendency in both fiber type-I content (p=0.14) and mitochondrial mass (p=0.05), but the effect of passages was not significant. One explanation might be because the differentiation capacity is reduced with progressive passaging, which affects the expression of fiber type-I, as fiber type-I is dependent on an adequate differentiations process (myoblasts capacity to fuse with each other). However, these differences were not significant, thus indicating that differences might not be observed before later passages or that the gap between the passages is too small. The slightly reduction of mitochondrial mass may be a cause of incomplete fiber
type-I expression with increased passages, since fiber type-I is more oxidative and contains more mitochondria [34]. Lipid content in myotubes did not show a decreasing pattern through multiple passaging indicating that this is a well-preserved characteristic of myotubes. Furthermore, correlations analysis between lipid content at passage 4 versus passage 5 and passage 6 showed consistency in the lipid content. However, this relationship was not preserved at passage 7 (passage 4 vs. passage 7). These results can possibly be explained by the gradually impaired lipid metabolism with increasing passages which has recently been described by Nehlin et al. [54]. Thus, this might be the cause of reduced lipid turnover with multiple passaging of human primary myotubes. It is important to note that no fatty acids were added to the culture media (growth and differentiation media) used for proliferating and differentiating of the skeletal muscle cells. Further research is needed to determine the effect of progressive passages on lipid content when lipid availability in the media is not a limiting factor.

Overall, no passage effect was observed on fiber type-I, IMCL and mitochondrial mass through passage 4 to passage 7. However, a slight reduction was observed for type-I fibers and mitochondrial mass with increased passages. This suggests that a possible passage effect is not determined before at later passages. Further studies are necessary to clarify the effect of passaging of myotubes on metabolic properties.
5 Conclusion

In conclusion, myotubes from athletes had higher amount of fiber type-I, lipid content and mitochondrial mass compared to myotubes cultured from lean and T2DM subjects. This relation has also been shown in skeletal muscle tissue. These findings were supported by distinctive correlation analysis performed on myotubes (fiber type-I and lipid content) versus in vivo parameters measured clinically, indicating that the donors phenotype was also preserved in in vitro cell culture model. No relationship was observed between lipid content measured in myotubes and IMCL measured in tissue. Interestingly, lipid content measured in myotubes was positively associated with clinical phenotypes such as physical fitness, fiber type-I and insulin sensitivity, suggesting that a high lipid content measured in myotubes reflected better physical fitness and higher insulin sensitivity. Four consecutive cell passages (passage 4 to passage 7) of primary myotubes did not have any effect on lipid content in the cells. However, fiber type-I content and mitochondrial mass showed a decreasing trend with progressive passaging of myotubes.
6 References


References


7 Appendix

7.1 Cell seeding and cell proliferation media

7.1.1

10% Growth media

500ml Dulbecco’s Modified Eagle Medium Low Glucose with L-glutamin (DMEM)
3,33ml 7,5% Bovine serum albumin (BSA)
500ul Gentamicin
50ml Fetal bovine serum (FBS)
500ul Dexamethasone
5ml Fetuin
100ul Fungizone
500ul Human epidermal growth factor (haEGF)

7.1.2

16% Growth media

500ml Dulbecco’s Modified Eagle Medium Low Glucose with L-glutamin (DMEM)
3,33ml 7,5% Bovine serum albumin (BSA)
500ul Gentamicin
80ml Fetal bovine serum (FBS)
500ul Dexamethasone
5ml Fetuin
100ul Fungizone
500ul Human epidermal growth factor (haEGF)
Appendix

7.1.3

10% dimethyl sulfoxide (DMSO) solution freezing liquid

  18ml 16% growth media
  2ml DMSO

7.1.4

2% Differentiations media

  500ml Minimum Essential Media with Glutamax (MEM)
  10ml Fetal bovine serum (FBS)
  10ml Penicillin-streptomycin
  5ml Fetuin
7.2 Immunohistochemistry reagents

7.2.1

0.1\% Saponin solution

*Stock Saponin 10%:*

10ml PBS

1g Saponin

500ul Stock Saponin 10%

5ml PBS

7.2.2

PBS

100ml 10xPBS

900ml milli-q water

7.2.3

3\% BSA

15g BSA

500ml PBS

7.2.4

0.5\% BSA

2.5g BSA

500ml PBS
7.2.5

Primary antibody MHC-I solution 1:100
- 200ul primary antibody MHC-I mouse
- 20ml PBS
- 3.33ml 3% BSA (0.5% BSA concentration)
- 20ul 10% Saponin stock

7.2.6

Mitochondria solution 1:100
- 200ul Mitochondria antibody
- 20ml PBS
- 3.33ml 3% BSA (0.5% BSA concentration)
- 20ul 10% Saponin stock (0.1% Saponin concentration)

7.2.7

0.05% Tween 20
- 1ml Tween 20
- 2000ml PBS

7.2.8

Secondary antibody solution 1:200
- 100ul secondary antibody 680 mouse Alexaflour
- 20ml PBS
- 3.33ml 3% BSA (0.5% BSA concentration)
- 20ul 10% Saponin stock (0.1% Saponin concentration)

7.2.9

Bodipy solution 1:100
- 500ul Bodipy
- 50ml PBS
Appendix

7.2.10

**DAPI solution 1:10000**
- 6μl DAPI
- 60ml PBS
7.3 Mitotracker reagents

7.3.1

Mitotracker solution

*Solution A:*
74.4 ul DMSO
1 tube of mitotracker green

*Solution B:*
10 ul solution A
90 ul DMEM

*Solution C:*
60 ul solution B
60 ml DMEM
Appendix

7.4 Western immunoblotting reagents

7.4.1

Protein buffer
  1ml RIPA
  10ul phosphatase 1
  10ul phosphatase 2
  10ul protease inhibitor

7.4.2

BCA solution 1:50
  100ul BCA protein assay reagent B
  5ml BCA protein assay reagent A

7.4.3

Blocking buffer
  50ml Odyssey blocking buffer
  50ml TBS-T

7.4.4

MHC-I solution 1:1000 and GAPDH solution 1:1000
  50ul antibody
  50ul GAPDH
  25ml blocking buffer
  25ml TBS
Appendix

7.4.5

**TBS-T**

- 200ml TBS
- 1800ml rinsed water
- 2μl Tween 20

7.4.6

**Secondary antibody solution 1:7500**

- 7μl antibody
- 50ml TBS-T

7.4.7

**TBS**

- 200ml TBS
- 1800ml rinsed water
7.5 Experiment: Real time Polymerase chain reaction – RNA

RNA harvesting

0.25% trypsin and TNS were added the same way as for the freeze down (see section 2.3.3 – Growing and differentiation of muscle cells into mytobues) and centrifuged. 1ml of Trizol was added to the cell pellet and mixed evenly. The cell suspension was transferred to an eppendorf vial, snap frozen in liquid nitrogen and stored at -80°C.

Purification of total RNA

As described previously [66], purification of total RNA was performed by using RNeasy® mini kit from Qiagen (cat#74104, Qiagen). In brief, harvest RNA was thawed and added 500µl phase separation reagent 1-bromo-3-chloropopane (BCP) for 5 minutes. The sample was spun down at 14000 rpm for 15 minutes at 4°C. The supernatant was transferred to a new eppendorf tube and additional 500µl BCP was added. This was spun down for 2 minutes at 4°C and 14000 rpm. The supernatant was transferred to a new eppendorf tube and added equally amount of 70% ethanol. This solution was transferred to RNeasy column with a catch tube and spun at 13 4000 rpm for 20 seconds at room temperature. RNeasy column was transferred to a new catch tube. 700µl RW1 buffer was added and spun at 13 4000 rpm for 20 seconds at room temperature. 500µl RPE buffer was added and spun at the same conditions as the previous spin. This step was performed two times where the final spin down lasted 3 minutes. RNeasy column was transferred to a new catch tube and spun on maximum speed for 1 minute. RNeasy column was placed into a new eppendorf vial and eluted with 60µl RNase free water. This was spun at 13 400 rpm for 1 minute at room temperature. RNA quantity was determined using a ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientific). RNA was stored at -80°C.

Real time PCR

Real time PCR was not performed because lack of time.