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Effect of Serial Cell Passaging in the Retention of Fiber Type and Mitochondrial Content in Primary Human Myotubes

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Abstract

Objective—The purpose of the study was to determine the effects of passaging on retention of donor phenotypic characteristics in primary human myotubes.

Methods—We established primary muscle cultures and serial passaged myotubes from physically active, sedentary lean, and individuals with type 2 diabetes (T2D). We measured maximal ATP synthesis capacity (ATPmax) and resting ATP flux (ATPase) *in vivo* by ^{31}P magnetic resonance spectroscopy, type-I fibers and intramyocelluar lipid (IMCL) in *vastus lateralis* tissue using immunohistochemistry techniques, and oxidative phosphorylation complexes (OXPHOS) by western immunoblotting. Similar *in vitro* measures for lipid and type-I fibers were made in myotubes, along with mitochondrial content measured by Mitotracker.

Results—Passage 4 and 5 measures for myotubes correlated positively with *in vivo* measurements for percent type-I fibers (P4: r=0.62, p=0.02; P5: r=0.69, p=0.01), ATPmax (P4: r=0.54, p=0.03; P5: r=0.47, p=0.05), and OXPHOS (P4: r=0.66, p=0.04; P5: r=0.77, p=0.006). No correlations were observed for IMCL. However, passage 4 measures for myotubes correlated with passage 5 measures for percent type-I fibers (r=0.70, p=0.01), IMCL (r=0.89, p<0.001), and mitochondrial content (r=0.51, p=0.03).

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Conclusions—Myotubes through the first two passages following immunopurification (referred to as passage 4 and 5) reflect mitochondrial and type-I fiber content *in vivo* phenotype of the donor.

Keywords

Intramyocellular lipid; type 2 diabetes; athlete's paradox; ectopic lipid

Introduction

It has long been held that skeletal muscle tissue is one of the primary targets for the pathogenesis of insulin resistance. In conditions of insulin resistance, such as type 2 diabetes (T2D), skeletal muscle displays reduced lipid oxidation[1], altered mitochondrial function[2,3], and increased lipid accumulation[4]. Aerobic exercise has been shown to improve both lipid oxidation and mitochondrial function[5]. But given the revelation of the athlete's paradox[6], heightened lipid stores along with increased skeletal muscle insulin sensitivity remain unsolved.

Studies into alterations in skeletal muscle metabolism have been greatly enhanced with the use of *in vitro* cell culture experiments using skeletal muscle myotubes[7,8]. Stable cell lines, such as C2C12 mouse myoblasts[9,10] and L6 rat myoblasts[11,12], have provided a valuable and routine sources for muscle-related experimentation. However, given the fact that these cell lines are not from human sources and are immortal, human primary skeletal muscle myotubes obviously offer a more powerful and relevant model to study skeletal muscle metabolism *in vitro*, especially with heterogeneous donor phenotypes. Importantly, what makes human primary muscle culture so advantageous is the fact that they have repeatedly been shown to preserve several phenotypic characteristics of their donor[13,14].

Unlike stable cell lines, use of human primary myotubes are limited by the number of passages it can be used[15]. It has been shown in other primary cell cultures[16-18], in rat primary skeletal muscle cultures[19], and in human skeletal muscle cultures[20] that the effect of passaging can alter the original characteristics of the cells from when they were originally cultured. However, to the best of our knowledge we are not aware of any studies that have examined the effect of human skeletal muscle myotube passaging on the preservation of phenotypic characteristics from distant passaging to initial culturing, nor have studies examined the effect of passaging on the retention of skeletal muscle donor phenotype characteristics. Here, we endeavored, therefore, to investigate 1) whether *in vivo* mitochondrial and fiber type characteristics of donors are retained *in vitro* in cultured human myotubes and 2) the effect of increased passages of human myotubes on *in vitro* lipid, mitochondrial and fiber type measurements using physically active, sedentary lean and T2D donors. We hypothesized that the increased number of passaging will impact negatively on both lipid and mitochondrial content in human myotubes. Additionally, this decrease in lipid and mitochondrial content will affect the correlation of lipid and mitochondrial parameters measured in donors *in vivo*.

Methods and Procedures

Participant Recruitment and Clinical Phenotypic Assessments

Six physically active, 6 sedentary lean, and 6 donors with T2D were recruited into this study. Participants were enrolled in with or without T2D, age 20-40 years and BMI 20-30 kg/m^2 (NCT00402012 and NCT00401791). Volunteers with chronic illnesses such as heart disease, hypothyroidism, renal, lung and liver diseases were excluded. Study was approved by the Institutional Review Board of Pennington Biomedical research center and participants provided written informed consent. Physical activity level was assessed from a 7-day physical activity recall questionnaire and a tri-axial accelerometer in healthy donors but not in donors with T2DM. Physical activity level (PAL; total daily energy expenditure/resting metabolic rate) was calculated and donors were classified as sedentary healthy controls (PAL<1.4) or active (PAL>1.6). All donors consumed a provided standard American diet (15:30-35:50-55% protein:fat:carbohydrate) for 3 days before admission to our inpatient clinic. Clinical anthropometric and metabolic phenotypic characteristics of study participants were performed as follows: Body weight was measured in a gown after voiding; waist circumference in the standing position and height with a calibrated stadiometer; body composition was assessed using dual x-ray absorptiometry (DXA, QDR4500A; Hologics, Waltham, MA); basal (ATPase) and maximal (ATPmax) *in vivo* assessment of skeletal muscle mitochondrial ATP production were performed under magnetic resonance spectroscopy (3T Signa Excite MRI; General Electric, Milwaukee, WI) as previously described[3]; and insulin sensitivity measured by euglycemic-hyperinsulinemic clamps were performed as previously described[3] using an insulin infusion of 80 mU/min/m^2, and glucose disposal rate (GDR) was normalized by estimated mean body size (EMBS; kg fat-free mass [FFM]+17.7)[21].

Skeletal Muscle Biopsy and Muscle Tissue Measures

After an overnight fast and local anesthesia (lidocaine/bupivicaine), skeletal muscle samples for immunohistochemistry, protein content and cell cultures[7] were collected from the *vastus lateralis* using the Bergstrom technique with suction. Intramyocellular lipid (IMCL) and fiber type was measured by immunohistochemistry performed on 12 micron sections using bodipy green 493/503 (Invitrogen molecular probe, CA) along with mouse monoclonal antibody specific for slow-twitch muscle (MAB1628; Chemicon, Temecula, CA) and a monoclonal antibody to laminin (AB2500, Abcam Inc, Cambridge, MA). Images were captured using confocal microscope (Leica SP5, Leica, Bannockburn, USA) and type-I fibers were counted to determine fiber type[3,7]. Lipid was measured in myotubes cultured from *vastus lateralis* muscle biopsies, using the exact same immunohistochemisty technique. Lipid content in skeletal muscle was quantified by carefully identifying area inside the muscle fibers excluding extramyocellular lipid (EMCL). IMCL was quantified using the Sigma Scan Pro 5.0 software. Total OXPHOS content was measured using the MitoScience Human OXPHOS complex antibody cocktail (Cat no. ab110411) and was adjusted to GAPDH (Cat no. AB9484; AbCam, Cambridge, MA). Imaging and quantification of western blots was facilitated on the Odyssey infrared imaging system (LiCor, Lincoln, NE).

Primary Human Skeletal Muscle Culture and Passaging

Establishment of human primary muscle culture has been modified from protocols as previously described[22]. In this study, we defined passaging as the act of removing cells from its culture plate via use of trypsin-EDTA and re-plating the suspended culture into a new culture plate or freezing down in liquid nitrogen. One half of suspended cultures were used for plating the subsequent passage, and one half of cells were frozen down for cryopreservation. All of our initial experiments were performed at passage 4 (P4, the first passage with formed myotubes). This is due to the realistic manner of collecting primary human myotubes from study participants. The initial cultures from human biopsies were performed in what we refer to as passage 0 (P0), in a collagen coated T-25 plate (Thermo Scientific, Waltham, MA). Here, we use the term P0 to reflect the fact that our initial culture from the biopsy tissue was not treated with trypsin-EDTA for initial plating. Once the skeletal muscle culture has been established and allowed to proliferate to ∼90% confluency, myoblasts are passaged and allowed to expand in T-75 plates (Corning Inc., Corning, NY) in order to ensure that sufficient culture material is obtained (P1). Once confluent, cell were then frozen down and stored in liquid nitrogen until all donors for this study had their primary muscle culture collected. Once all muscle cultures had been obtained from study donors, cell were thawed and plated for expansion and immuno-sorting to select myoblast progenitor cells (P2). Myoblast skeletal muscle progenitor cells were immuno-sorted using the 5.1H11 antibody provided by the Hybridoma Bank (University of Iowa) and the MACS cell sorting column system (Miltenyi Biotec, Auburn, CA). Following sorting, cells were allowed to proliferated once again before being frozen down and stored in liquid nitrogen for study related experimentation (P3). Experiments on all donors began simultaneously following re-proliferation of P4 cells after having been thawed and re-plated. P4 cells were divided into those plated for experimentation purposes and those plated for the purpose of expansion and re-plating into the next passage. Cells destined for repassaging were not differentiated into myotubes, but were maintained as myoblasts. Subsequent passages 5, 6, and 7, were performed in this same manner. All experiments were performed on differentiated myotubes from each passage.

Lipid Content in Primary Myotubes

Myotubes were stained as previously described[23]. Lipid droplets and nucleus were stained using bodipy 493/503 (Invitrogen Molecular Probes) followed by DAPI (Sigma-Aldrich, St. Louis, MO). Lipid content was quantified, as described above, with the cyan-green channel using Softamax PRO5 FLEX station (Molecular devices, Sunnyvale, CA).

Mitochondrial Content in Primary Myotubes

Mitochondrial mass was determined by measuring fluorescence intensity of the cells after incubation with Mitotracker Green (Molecular Probes, Invitrogen, Eugene, OR) using a spectrometer model LS50B (PerkinElmer, East Lyme, CT, USA) with excitation and emission wavelengths of 490 and 516 nm, respectively. Mitotracker Green probe preferentially accumulates in mitochondria regardless of the mitochondrial membrane potential and thus provides an accurate assessment of mitochondrial mass[24].

Statistical Analysis

All statistical analyses were performed using GraphPad Prism, version 5.0 (*GraphPad* Software Inc, La Jolla, CA). All values are presented as means ± SEM. One-way ANOVA with Tukey post hoc test was used to determine any group difference (active, sedentary lean, and T2D; Table 1), and a two-way ANOVA with Tukey post-hoc test was used to determine differences between group and myotube passaging (Figure 1). Myotubes characteristics and clinical phenotypes were compared by Pearson correlation analysis and nonparametric Spearman's rho analysis, when applicable. A $p < 0.05$ was considered significant.

Results

Participant Characteristics

Participants with T2D significantly differed from both physically active and healthy sedentary lean subjects in terms of age, body weight, BMI, percent body fat, fasting glucose, and fasting insulin (Table 1). Furthermore, participants with T2D and sedentary lean subjects had lower glucose disposal rate (GDR), ATPmax, and ATPase levels compared to the physically active group (Table 1).

Effects of Passaging on Type-I Fibers, lipid, and Mitochondrial Content in Primary Myotubes

Differences in type-I fiber content in myotubes assessed by 2-way ANOVA was significant for group ($p = 0.01$). Myotubes cultured from active participants had higher levels of type-I fibers compared to that of T2D participants in P4 and P5, and lean participants in P5 (all $p<0.05$, Figure 1A). Type-I fibers were detected in myotubes cultured from all physically active donors, throughout all passages (P4-P7). For myotubes from lean donors, type-I fibers were detected in 4 individuals for both P4 and P5. By P6, only one lean individual's myotubes had detectable type-I fibers, and by P7 no lean individuals had detectable type-I fibers. For P4, type-I fibers were detected in myotubes from 5 T2D individuals. In P5 and P7, only 4 T2D myotubes had detectable type-I fibers. However, in P6, all 6 T2D individuals had detectable type-I fibers in their myotubes, albeit, barely datectable in 2 of the 6 individuals. Two-way ANOVA comparing lipid content in myotubes was significant for group ($p=0.003$), but not for passaging/time ($p = 0.08$). Myotubes from active donors had greater lipid content compared to lean and T2D myotubes for P4 and P5 (all $p<0.05$), but the significance of these relationships disappeared by P6 (Figure 1B). Two-way ANOVA for mitochondrial mass, assessed in live myotube by Mitotracker green dye, was found to be significant for group ($p<0.001$) and for passaging ($p=0.003$). Mitochondrial mass was significantly greater in myotubes from active donors over leans in both P4 and P6 (all $p<0.05$, Figure 1C). In P5, both active and T2D myotubes had significantly higher mitochondrial mass compared to lean myotubes (both $p<0.05$, Figure 1C). The relationships between myotubes from each cohort for mitochondrial mass disappeared in P7, and levels of mitochondrial mass in active and T2D myotubes were significantly lower in P7 as compared to P4 (both $p<0.05$, Figure 1C).

Correlations between *in vitro* measurements in myotubes with *in vivo* measures in all donors – Effects of Passaging

The percent of type-I fibers measured in the *vastus lateralis* muscle tissue from all donors with detectable type-I fibers correlated positively with type-I fibers measured in myotubes in P4 (r=0.62, p=0.02, n=14) and P5 (r=0.69, p=0.01, n=14), but not in P6 or P7 (Table 2). There were no correlations between lipid measured in myotubes and IMCL measured in tissue (Table 2). Total OXPHOS content measured in muscle tissue correlated with mitochondrial mass measured in myotubes in P4 (r=0.66, p=0.04, n=11), in P5 (r=0.77, p=0.006, n=11), and in P6 (r=0.81, p=0.004, n=11), but not in P7 (Table 2). *In vivo* maximal mitochondrial ATP synthesis rate (ATPmax) correlated with myotube mitochondrial mass measures in P4 (r = 0.54, p = 0.03, n = 14) and in P5 (r=0.47, p=0.05, n=14), but not with P6 or P7 (Table 2). *In vivo* resting mitochondrial capacity (ATPase) tended to have a positive correlation with myotube mitochondrial mass in P4 (r=0.58, p=0.06, n=14) and P5 (r=0.45, p=0.10, n=14), and correlated significantly in P6 (r=0.60, p=0.03, n=14), but not in P7 (Table 2).

Comparisons between P4 type-I fiber measures in myotubes positively correlated with those donors, who expressed detectable type-I fiber measures, in P5 (r=0.70, p=0.01; n=13), P6 (r=0.81, p=0.002; n=11), and P7 (r=0.79, p=0.01, n=10) (Table 3). Lipid content in P4 positively correlated with lipid measures in P5 (r=0.89, p<0.001, n=15) and P6 (r=0.85, p<0.001, n=13), but not in P7 (Table 3). Finally, mitochondrial mass in P4 correlated positively with mitochondrial mass in P5 (r=0.51, p=0.03, n=17), but not with P6 or P7 (Table 3).

Discussion

Our study shows that both mitochondrial content and type-I fibers are preserved in human primary skeletal muscle myotube culture, which mirror the phenotypic characteristics of the donor. We have shown that the passaging of primary myotubes does in fact have an effect on this retention of phenotype. This study offers evidence that primary myotubes can be an effective tool for performing *in vitro* experiments in donors of different inherent phenotypic characteristics related to differences in mitochondrial function and fiber type, both of which have been shown to vary with regards to insulin resistance and T2D[2,25,26]. This study also shows that the effect of distant passaging of primary myotubes does influence this retention of phenotype, and thus considerations of these effects must be made when embarking on experiments using human myotubes.

The disparities between inherent, genetic factors and environmental, circulating factors that influence the pathogenesis of skeletal muscle insulin resistance still remain a mystery[27,28]. A prime example being that of the athlete's paradox[6], whereby both insulin resistant T2D individuals and endurance trained athletes possess high levels of IMCL *in vivo* yet differ greatly along the insulin sensitivity spectrum. Prior reports investigating lipid droplet coat proteins comparing myotubes from leans verses T2Ds showed that there was no difference in basal protein levels of perilipin 2, perilipin 3, perilipin 5, or adipose triglyceride lipase (ATGL)[29]. Kitzmann et al showed that there was no difference in basal activity for 5′ adenosine monophosphate-activated protein kinase (AMPK) or its downstream target acetyl-

CoA carboxylase (ACC) in myotubes taken from controls verses T2Ds[30]. Similarly, gene expression levels of lipid metabolism pathway targets in myotubes cultured from non-diabetic and donors with T2Ds showed no differences in the basal mRNA expression of beta-hydroxyacyl-coA dehydrogenase (β-HAD), citrate synthase, carnitine-palmitoyl transferase 1 (CPT1). It was further shown that in the presence of a high level of palmitate AMPK and ACC activity were upregulated in non-diabetic controls but not in T2Ds. A study by Wensaas et al. compared treatments of various lipid species to myotubes taken from both obese non-diabetic individuals and T2Ds showed that differences in the mRNA levels of diacylglycerol-acyl transferase 2 (DGAT) and peroxisome proliferating activator receptor gamma, co-activator 1 alpha (PGC1a) occurred only when lipids were introduced[31].

We previously reported that primary myotubes do not mirror the similar lipid levels as their donors *in vivo*[13] when cells are cultured without additional lipid in the media. Our present study also confirms that lipid levels remain elevated in myotubes cultured from physically active donors in earlier passages; additionally, lipid levels from all cohorts remained correlated with each other from P4 through P6. We utilized the same levels of lipid within the culture media to treat all groups similarly. Overall, this suggests that lipid retention and accumulation in an *in vitro*/*ex vivo* setting is more influenced by inherent genetic and/or epigenetic properties of the tissue rather than the *in vivo* milieu. Since the aforementioned studies have shown no differences in basal expression or activity related to lipid metabolic pathways in myotubes taken from both sedentary lean individuals and individuals with T2D, this may, in part, explain why under basal conditions we saw no differences in lipid accumulation between these two group. Additionally, our studies, to the best of our knowledge, appear the be the first studies to compare lipid accumulation in myotubes taken from physically active donors to myotubes taken from sedentary leans and T2Ds. Unlike physically active myotubes, T2D muscle in the *in vivo* setting is perhaps more influenced by circulating factors that result in its retention and accumulation of lipid. Further investigations using primary myotubes from donors with T2D can perhaps resolve the disparities between IMCL content and ultimately the athlete's paradox.

Aside from IMCL, both fiber type[32] and mitochondrial content/function[2] have been shown to affect insulin sensitivity. Extensive work has shown, regardless of cause or consequence, that mitochondrial content/function is involved in insulin sensitivity and is altered in muscle from individuals with T2D[3]. Our study shows that mitochondrial content in living myotube cultures, as assessed by Mitotracker, is positively correlated with live, *in vivo* measures of mitochondrial capacity and with muscle tissue protein content of the oxidative phosphorylation complexes (OXPHOS). This shows that primary myotubes can be used to perform *in vitro* mitochondrial related experiments that can be translatable to clinical endpoints because mitochondrial content in myotubes should adequately reflect the mitochondrial content/function of the donor. Additionally, type-I fibers have been shown to be diminished in individuals with T2D[32,33]. To the best of our knowledge, our lab group has been the first successful lab to adequately identify type-I fibers in primary muscle cultures using immunohistochemistry technique[34]. This insight along with the results of our study showing retention of type-I fiber content reflective of the donor provides evidence that fiber type related and fiber type specific culture experiments could be feasible, especially to labs

that do not have clinical access to muscle biopsy specimens. Currently, it is unclear why the content of type-I positive myotubes diminish with passaging in both the lean and T2D cohorts. Our results show that type-I content in myotubes cultured from physically active donors were higher compared to that of lean (passage 4 and 5) and T2D donors (passage 4). Further, the content of type-I fibers appears to be maintained throughout passaging in the active donors. During our experiments, we have made every effort to treat all cultures under identical experimental conditions, and ensured that fused, mature myotubes were present in cultures before experimental techniques were undertaken. Our understanding of this phenomenon suggests that something relating to type-I fiber retention is maintained in myotubes from active donors, but is not present within sedentary lean donors and donors with T2D. Future investigations would be needed to determine this phenomenon.

Currently, there is disagreement within the literature concerning the duration of differentiation for human primary myotubes. Some studies have reported that cultures were allowed to differentiate for 8 days[22,30], some for 7 days[7,35], while others have allowed their cultures to differentiate for as little as 4 days[31] to 5 days[36]. We used 7 days to differentiate our cultures because this duration resulted in visual confirmation of fused myotubes in all cultures regardless of passage number or group. Though our study here is not aimed at specifically determining alterations in lipid retention, mitochondrial content, or fiber type composition as differentiation progresses from day 1 to day 7, the end-point aims of our study should be comparable given that all cultures were treated equally, simultaneously, and allowed the same time to differentiate. Further studies would be interesting and necessary to explore alterations in these patters as differentiation progresses through the different cohorts.

The major strengths of our study lay in the detailed *in vivo* clinical phenotyping measures alongside the *in vitro* measure of metabolic parameters using human primary cell cultures from the same donors. Similarly, we performed the same protocols for IHC measures of fiber typing and IMCL to both myotubes and muscle tissue. Although we were not able to obtain the exact same measure of mitochondrial content in myotubes as in skeletal muscle tissue (namely because Mitotracker dye only works on live cells, which would be unobtainable in muscle tissue), we did obtain three different measures related to mitochondrial function/content, two of which relate to the living cells *in vivo* using magnetic resonance spectroscopy. The careful, clinical control of our phenotypic measures suggests that the relationships we reported between the *in vivo* characteristics of participants and their donated myotubes are sound.

Unlike cross-sectional analysis of fixed skeletal muscle tissue, IHC analysis of myotubes requires the examination of the entire culture plate of myotubes. Thus, delineation and normalization of data obtained per myotube remains challenging. However, use of DAPI nuclear staining to normalize data has been widely used and accepted[14,20,39]. We made every effort to ensure proper seeding of culture plates with the exact same number of cells for our experiments. Furthermore, the quantification of DAPI among cohorts and among passages remained unchanged for our experiments (data not shown). A second limitation with our study was the age difference between our T2D group to the other two groups. However, we previously reported that when covariate analysis of this data was performed, age was not found to be a significant factor[3]. An additional limitation of our study was the

methods by which we were able to perform our experiments across all donors simultaneously. Due to the nature of human clinical research, it would have been logistically impossible to obtain biopsies from all donors on this study on the same day, and perform the initial phases of culturing simultaneously such that P0 would initiate for all participants on the same day. Therefore, in order to reduce experimental error by performing the experiments as cultures were obtained, it is of course unknown whether the duration of cryopreservation of the cells might have an impact on our results. However, this was necessary and in line with other primary human cell culture experiments that purchase commercially available myotubes where by myotubes are collected and frozen for different lengths of time.

To conclude, primary human myotubes offer a powerful platform for *in vitro* experiments in research related to mitochondrial function and the pathogenesis of type 2 diabetes. This is due to the fact that primary myotubes retain the phenotype of their donors. Disparities between *in vivo* environmental contributors as well as genetic/inherent contributors to skeletal muscle insulin sensitivity can now be examined confidently. Importantly though, our study does show that further passaging of primary myotubes does diminish the retainability of donor phenotype. Our recommendation is that following myoblast immunosorting, *in vitro* experiments should be relegated only to the first and second passages after sorting (what we have here referred to as passage 4 and passage 5). Our recommendation is based on the fact that *in vivo* and *in vitro* fiber typing and mitochondrial measures positivity correlate, and that there are significant positive correlations between passage 4 and passage 5 for type-I fiber content, lipid, and mitochondrial content. Furthermore, relationships and values of type-I fibers, mitochondrial content and lipid began to differ by the time passage 6 was reached.

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J.D.C. conceived the experiment, performed experiments, analyzed data, and wrote the manuscript; C.K.M. performed experiments, contributed to discussion and reviewed/edited the manuscript; A.C.R. conceived of the experiment, contributed to discussion, and reviewed/edited the manuscript; E.R. conceived of the experiment, contributed to the discussion, and reviewed/edited the manuscript; S.R.S. contributed to discussion and reviewed/edited the manuscript; S.B. conceived the experiment, performed experiments, analyzed data, and reviewed/edited the manuscript. All authors gave final approval of the manuscript prior to submission. J.D.C. is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Gaster M. Reduced lipid oxidation in myotubes established from obese and type 2 diabetic subjects. Biochem Biophys Res Commun. 2009; 382:766–770. [PubMed: 19324004]
2. Mogensen M, et al. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes. 2007; 56:1592–1599. [PubMed: 17351150]
3. Bajpeyi S, et al. Skeletal muscle mitochondrial capacity and insulin resistance in type 2 diabetes. J Clin Endocrinol Metab. 2011; 96:1160–1168. [PubMed: 21307136]
4. Krssak M, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. Diabetologia. 1999; 42:113–116. [PubMed: 10027589]
5. Sparks LM, et al. Nine months of combined training improves ex vivo skeletal muscle metabolism in individuals with type 2 diabetes. J Clin Endocrinol Metab. 2013; 98:1694–1702. [PubMed: 23463651]
6. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. J Clin Endocrinol Metab. 2001; 86:5755–5761. [PubMed: 11739435]
7. Covington JD, et al. Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and are associated with fat oxidation. PLoS One. 2014; 9:e91675. [PubMed: 24632837]
8. Aas V, et al. Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. Acta Physiol Scand. 2005; 183:31–41. [PubMed: 15654918]
9. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature. 1977; 270:725–727. [PubMed: 563524]
10. Blau HM, et al. Plasticity of the differentiated state. Science. 1985; 230:758–766. [PubMed: 2414846]
11. Yaffe D. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci U S A. 1968; 61:477–483. [PubMed: 5245982]
12. Richler C, Yaffe D. The in vitro cultivation and differentiation capacities of myogenic cell lines. Developmental biology. 1970; 23:1–22. [PubMed: 5481965]
13. Bajpeyi S, et al. Lipid in skeletal muscle myotubes is associated to the donors' insulin sensitivity and physical activity phenotypes. Obesity (Silver Spring). 2014; 22:426–434. [PubMed: 23818429]
14. Corpeleijn E, et al. Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids. Am J Physiol Endocrinol Metab. 2010; 299:E14–22. [PubMed: 20442319]
15. Clark WA, Decker ML, Behnke-Barclay M, Janes DM, Decker RS. Cell contact as an independent factor modulating cardiac myocyte hypertrophy and survival in long-term primary culture. Journal of molecular and cellular cardiology. 1998; 30:139–155. [PubMed: 9500872]
16. Wall ME, Bernacki SH, Loboa EG. Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells. Tissue engineering. 2007; 13:1291–1298. [PubMed: 17518709]
17. Tezel TH, Del Priore LV. Serum-free media for culturing and serial-passaging of adult human retinal pigment epithelium. Experimental eye research. 1998; 66:807–815. [PubMed: 9657913]
18. Zimmermann T, et al. Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture--primary culture cells markedly differ from fourth-passage cells. Arthritis research. 2001; 3:72–76. [PubMed: 11178129]
19. Machida S, Spangenburg EE, Booth FW. Primary rat muscle progenitor cells have decreased proliferation and myotube formation during passages. Cell proliferation. 2004; 37:267–277. [PubMed: 15245563]
20. Nehlin JO, Just M, Rustan AC, Gaster M. Human myotubes from myoblast cultures undergoing senescence exhibit defects in glucose and lipid metabolism. Biogerontology. 2011; 12:349–365. [PubMed: 21512720]
21. Lillioja S, Bogardus C. Obesity and insulin resistance: lessons learned from the Pima Indians. Diabetes/metabolism reviews. 1988; 4:517–540. [PubMed: 3061759]

22. Gaster M, Rustan AC, Beck-Nielsen H. Differential utilization of saturated palmitate and unsaturated oleate: evidence from cultured myotubes. Diabetes. 2005; 54:648–656. [PubMed: 15734839]
23. Ohsaki Y, Maeda T, Fujimoto T. Fixation and permeabilization protocol is critical for the immunolabeling of lipid droplet proteins. Histochem Cell Biol. 2005; 124:445–452. [PubMed: 16151827]
24. Presley AD, Fuller KM, Arriaga EA. MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection. Journal of chromatography B Analytical technologies in the biomedical and life sciences. 2003; 793:141–150. [PubMed: 12880861]
25. Stuart CA, et al. Slow-twitch fiber proportion in skeletal muscle correlates with insulin responsiveness. J Clin Endocrinol Metab. 2013; 98:2027–2036. [PubMed: 23515448]
26. Albers PH, et al. Human muscle fiber type specific insulin signaling - Impact of obesity and type 2 diabetes. Diabetes. 2014
27. Gaster M, Beck-Nielsen H. The reduced insulin-mediated glucose oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin--evidence from cultured myotubes. Biochim Biophys Acta. 2004; 1690:85–91. [PubMed: 15337174]
28. Gaster M, Rustan AC, Aas V, Beck-Nielsen H. Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. Diabetes. 2004; 53:542–548. [PubMed: 14988236]
29. Sparks LM, et al. Reduced incorporation of fatty acids into triacylglycerol in myotubes from obese individuals with type 2 diabetes. Diabetes. 2014; 63:1583–1593. [PubMed: 24487026]
30. Kitzmann M, et al. Abnormal metabolism flexibility in response to high palmitate concentrations in myotubes derived from obese type 2 diabetic patients. Biochim Biophys Acta. 2011; 1812:423–430. [PubMed: 21172433]
31. Wensaas AJ, et al. Fatty acid incubation of myotubes from humans with type 2 diabetes leads to enhanced release of beta-oxidation products because of impaired fatty acid oxidation: effects of tetradecylthioacetic acid and eicosapentaenoic acid. Diabetes. 2009; 58:527–535. [PubMed: 19066312]
32. Oberbach A, et al. Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. Diabetes Care. 2006; 29:895–900. [PubMed: 16567834]
33. Gaster M, Staehr P, Beck-Nielsen H, Schroder HD, Handberg A. GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 fiber disease? Diabetes. 2001; 50:1324–1329. [PubMed: 11375332]
34. Aas V, et al. Are cultured human myotubes far from home? Cell Tissue Res. 2013
35. Aguer C, et al. Galactose enhances oxidative metabolism and reveals mitochondrial dysfunction in human primary muscle cells. PLoS One. 2011; 6:e28536. [PubMed: 22194845]
36. Engeli S, et al. Natriuretic peptides enhance the oxidative capacity of human skeletal muscle. J Clin Invest. 2012; 122:4675–4679. [PubMed: 23114600]
37. Bischoff R. Regeneration of single skeletal muscle fibers in vitro. The Anatomical record. 1975; 182:215–235. [PubMed: 168794]
38. Yaffe D, Feldman M. The Formation of Hybrid Multinucleated Muscle Fibers from Myoblasts of Different Genetic Origin. Developmental biology. 1965; 11:300–317. [PubMed: 14332576]
39. Gjelstad IM, et al. Expression of perilipins in human skeletal muscle in vitro and in vivo in relation to diet, exercise and energy balance. Archives of physiology and biochemistry. 2012; 118:22–30. [PubMed: 22117101]

What is already known about this subject

- Differences in skeletal muscle phenotype exist between sedentary, healthy individuals, active individuals, and individuals with type 2 diabetes
- Primary human myotubes provide a powerful and useful model to study these differences *in vitro*

What this study adds

- Primary myotubes from these cohorts reflect the *in vivo* phenotype of mitochondrial content and fiber type
- Following serial passaging, the retention of *in vivo* phenotypic characteristics is not reflected in later passages.

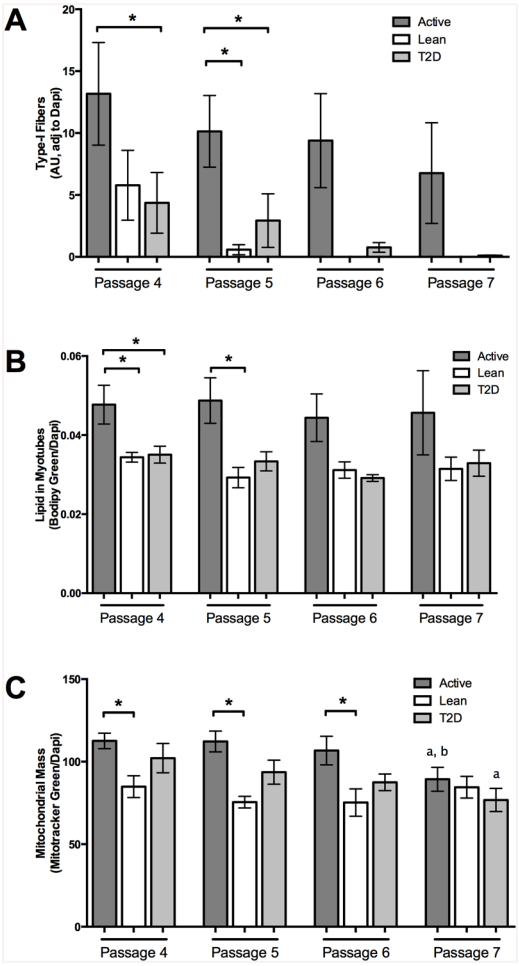

Figure 1.

A) Type-I fibers, **B)** Intramyocellular lipid content, and **C)** Mitochondrial mass between Physically Active, Sedentary Leans, and individuals with Type 2 Diabetes (T2D). * $p < 0.05$; $^{a}p < 0.05$ compared to passage 4; $^{b}p < 0.05$ compared to passage 5.

Table 1
Cross-sectional anthropometric and metabolic characteristics

	Active	Lean	T2DM
N (M/F)	6 (6/0)	6 (6/0)	6 (4/2)
Age (years)	23 ± 1	25 ± 1	43 ± 4[a,b]
Weight (kg)	77.6 ± 3.2	71.4 ± 4.2	110 ± 6.5[a,b]
BMI (kg/m2)	24.4 ± 0.9	22.9 ± 0.9	40.2 ± 2.2[a,b]
Body fat (%)	13.6 ± 1.2	19.7 ± 1.4	36.7 ± 3.4[a,b]
Fasting glucose (mmol/l)	4.8 ± 0.1	4.7 ± 0.2	6.9 ± 0.5[a,b]
Fasting insulin (μU/ml)	3.5 ± 0.9	8.1 ± 2.1	29.4 ± 3.3[a,b]
GDR (mg/min/EMBS)	12.0 ± 0.9	8.2 ± 1.0[a]	3.3 ± 0.7[a]
ATPmax (mM/sec)	1.1 ± 0.1	0.7 ± 0.1[a]	0.5 ± 0.1[a]
ATPase (μM/sec)	7.2 ± 0.8	5.3 ± 0.4	3.1 ± 0.6[a]
IMCL (AU)	15.4 ± 3.3	5.6 ± 2.1	9.9 ± 3.7
% Type-I Fibers	52.2 ± 16.1	36.1 ± 18.1	37.4 ± 15.7
OXPHOS (AU)	1.07 ± 0.26	0.51 ± 0.14[a]	0.37 ± 0.04[a]

Data are presented as mean±SEM;

[a] $p < 0.05$ from active participants.

[b] $p < 0.05$ from lean participants.

BMI, Body Mass Index; GDR, glucose disposal rate; EMBS, estimated mean body size; IMCL, intramyocellular lipid; OXPHOS, oxidative phosphorylation complexes as measured by western blotting.

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Table 2

Correlation coefficients between myotube measures and muscle tissue measures

Measures in Muscle Tissue	**Type-I fibers in Myotubes, AU (Fiber type-I antibody/DAPI)**							
Vastus Lateralis	**Passage 4**		**Passage 5**		**Passage 6**		**Passage 7**	
	R^2	**p value**	**R^2**	**p value**	**R^2**	**p value**	**R^2**	**p value**
Fiber type – I (%)	**0.39**	**0.02**	**0.48**	**0.01**	0.06	0.46	0.22	0.17
	Lipid in Myotubes (Bodipy Green/DAPI)							
	Passage 4		Passage 5		Passage 6		Passage 7	
	R^2	p value	R^2	p value	R^2	p value	R^2	p value
Intramyocellular Lipid (IMCL)	0.001	0.89	<0.001	0.91	<0.001	0.98	0.04	0.54
	Mitochondrial Mass in Myotubes (Mitotracker Green/DAPI)							
Mitochondrial content	Passage 4		Passage 5		Passage 6		Passage 7	
	R^2	p value	R^2	p value	R^2	p value	R^2	p value
Total OXPHOS	**0.44**	**0.04**	**0.59**	**0.006**	**0.66**	**0.004**	<0.001	0.95
ATPmax	**0.30**	**0.03**	**0.22**	**0.05**	0.11	0.22	0.01	0.71
ATPase	0.25	0.06	0.20	0.10	**0.37**	**0.03**	0.06	0.50

IMCL, intramyocellular lipid; OXPHOS, Oxidative Phosphorylation Complexes in Mitochondria.

Table 3

Correlations coefficients between passage 4 and further passages in myotubes

Measure in Passage 4	Passage 5		Passage 6		Passage 7	
	Type-I fiber					
	R^2	**p value**	**R^2**	**p value**	**R^2**	**p value**
Type-I fiber	**0.49**	**0.01**	**0.65**	**0.002**	**0.62**	**0.01**
	IMCL					
	R^2	p value	R^2	p value	R^2	p value
IMCL	**0.80**	**<0.001**	**0.73**	**<0.001**	0.26	0.09
	Mitochondrial Mass					
	R^2	p value	R^2	p value	R^2	p value
Mitochondrial Mass	**0.26**	**0.03**	0.08	0.32	0.07	0.40

IMCL, intramyocellular lipid.