Metabolic and Transcriptional Changes in Cultured Muscle Stem Cells from Low Birth Weight Subjects

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Context/Objective: Developmental programming of human muscle stem cells could in part explain why individuals born with low birth weight (LBW) have an increased risk of developing type 2 diabetes (T2D) later in life. We hypothesized that immature muscle stem cell functions including abnormal differentiation potential and metabolic function could link LBW with the risk of developing T2D.

Design/Settings/Participants: We recruited 23 young men with LBW and 16 age-matched control subjects with normal birth weight. Biopsies were obtained from vastus lateralis, and muscle stem cells were isolated and cultured into fully differentiated myotubes.

Main Outcome Measures: We studied glucose uptake, glucose transporters, insulin signaling, key transcriptional markers of myotube maturity, selected site-specific DNA methylation, and mitochondrial gene expression.

Results: We found reduced glucose uptake as well as decreased levels of glucose transporter-1 and -4 mRNA and of the Akt substrate of 160-kDa mRNA and protein in myotubes from LBW individuals compared with normal birth weight individuals. The myogenic differentiation markers, myogenin and myosin heavy chain 1 and 2, were decreased during late differentiation in LBW myotubes. Additionally, mRNA levels of the peroxisome proliferator-activated receptor-γ coactivator-1α and cytochrome c oxidase polypeptide 7A were reduced in LBW myotubes. Decreased gene expression was not explained by changes in DNA methylation levels.

Conclusion: We demonstrate transcriptional and metabolic alterations in cultured primary satellite cells isolated from LBW individuals after several cell divisions, pointing toward a retained intrinsic defect conserved in these myotubes. (J Clin Endocrinol Metab 101: 2254–2264, 2016)

Skeletal muscle plays a central role in metabolic health and is the predominant site of whole-body insulin-mediated glucose uptake and metabolism (1). A number of studies in low birth weight (LBW) individuals have found reduced insulin-stimulated muscle glucose uptake (2–4); decreased protein expression of key insulin signaling mol-

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Abbreviations: AS160, Akt substrate of 160 kDa; BMI, body mass index; CS, citrate synthase; FBS, fetal bovine serum; GLUT, glucose transporter; GSK3β, glycogen synthase kinase-3β; LBW, low birth weight; MHC, myosin heavy chain; MyoD, myogenin differentiation 1; NBW, normal birth weight; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; P5, penicillin-streptomycin; T2D, type 2 diabetes.
ecules, eg, p85, PKCζ, glucose transporter (GLUT) 4, and p110β in skeletal muscle (5); immature muscle fiber composition (6); as well as reduced peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and oxidative phosphorylation (OXPHOS) muscle gene expression (7). These alterations are not always reflected in whole body glucose and insulin metabolism at this stage of age (23–24 years of age). Nonetheless, these alterations may explain in part why LBW is associated with an increased risk of developing insulin resistance and type 2 diabetes (T2D) later in life (8, 9). However, it is unknown to what extent impairment of the LBW muscle occurs in vivo as a result of metabolic abnormalities or whether it is due to intrinsic impairments of muscle cellular functions preprogrammed by epigenetic mechanisms in fetal life.

Formation of skeletal muscle during fetal development is driven by myogenic progenitor cells (10). After birth, myogenic progenitor cells give rise to muscle stem cells, also termed satellite cells (11). Under normal conditions, satellite cells are in a quiescent state, but in response to trauma or muscle maintenance, they are activated and proliferate, and they subsequently differentiate and form new fibers or fuse to existing myofibers (12). Myogenesis is controlled by myogenic regulatory factors, including myogenin differentiation 1 (MyoD) and myogenin, which are muscle-specific transcription factors that regulate muscle-specific genes (13). Myosin heavy chain (MHC) is part of the contractile unit in muscle cells and is expressed late in the differentiation process when the myotubes have fused (14). This cooperative regulation allows the correct timing of the muscle-specific gene program, directing the differentiation of myoblasts into myotubes (15). Epigenetic mechanisms, including miRNAs, chromatin remodeling, and DNA methylation, may be involved in regulating myogenesis and myogenic regulatory factors (16). Epigenetic regulation may in turn be influenced by the fetal environment (7, 17, 18), potentially affecting muscle satellite cells and myogenesis.

Satellite cells isolated from T2D individuals and differentiated into myotubes display impaired glucose uptake (19, 20), insulin resistance (21), reduced lipid oxidation (22), as well as altered myogenic potential (23), suggesting that they retain their in vivo phenotype. A decreased expression of OXPHOS genes and PGC-1α has been demonstrated in skeletal muscle of T2D individuals, and it was suggested that impaired regulation of PGC-1α was linked to the development of peripheral insulin resistance by influencing mitochondrial function in these patients (24, 25). Furthermore, reduced expression of PGC-1α in muscle has previously been shown in twins with LBW (26) and in young singleton men with LBW after a high-fat overfeeding challenge (7).

In this study, we aim to compare differentiation potential and metabolic function in cultured muscle satellite cells isolated from LBW individuals and normal birth weight (NBW) controls.

**Subjects and Methods**

**Study design and human muscle precursor cell isolation**

This study was performed in accordance with the Declaration of Helsinki and approved by the regional ethical committee (H-A 2009–040 and H-D 2008–127). Twenty-three individuals born with LBW (birth weight below the 10th percentile) and 16 NBW individuals (birth weight in the 50th–90th percentile range) were recruited through The Danish National Birth Registry (17, 27, 28), based on birth weight data from Denmark at the time the study participants were born. All individuals were healthy lean male singletons born at term (maternal gestational week 39–41; no premature subjects were included in the study) with no family history of diabetes in two generations. Anthropometric measurements were obtained, and body composition was determined by dual-energy x-ray scanning. After an overnight fast, blood samples and skeletal muscle biopsies were obtained from the vastus lateralis muscle using a Bergström biopsy needle. Eight subjects participated in a study by Jørgensen et al (28), and 31 subjects in a study by Mortensen et al (27), both using the same stringent inclusion criteria. Participants included in the study by Mortensen et al (27) also underwent a 3-hour euglycemic-hyperinsulinemic clamp (80 mU·m⁻²·min⁻¹) after the overnight fast (unpublished data). The total number of participants in this study (39 LBW and NBW combined) was limited only by the availability of muscle biopsies from the participants in these studies. Human muscle precursor cells were isolated from each muscle biopsy as previously described (29). A preplating step was included to obtain an enriched population of satellite cells by removing fibroblasts that adhere more strongly to the plastic in the cell culture plate than satellite cells do. The cells were then divided into three fractions: one to detect myoblast purity, one for performing this cell study, and one for storage.

**Flow cytometry for determination of muscle precursor cell purity**

To assess the myogenic purity of the primary human skeletal muscle cell cultures (LBW, n = 23; NBW, n = 16), the expression of the cell surface markers CD56, CD31, and CD45 was measured using flow cytometry. Isolated cells were propagated in growth medium (HAM-F10 supplied with 20% fetal bovine serum [FBS], 1% penicillin-streptomycin [PS], and 1% fungizone) until 70–80% confluence in 5% CO₂, 37°C environment. Cells were detached using TrypLE and then washed twice in wash buffer (PBS containing 2% FBS and 0.01% NaN₃) and once in staining buffer (PBS containing 2% FBS, 1% human serum, and 0.01% NaN₃). Cells were stained with antihuman CD56-APC, CD31-PE, and CD45-BV421 (all BD Bioscience) for 20 minutes and subsequently washed three times in wash buffer. Data were acquired using a FACSFortessa (BD Biosciences). For compensation, single stain was used with one drop of negative control beads and antimouse IgG beads (BD Biosciences). Data analysis
was performed using Kaluza software version 1.2 (Beckman Coulter).

**In vitro cell studies**

For all differentiation experiments, the isolated muscle precursor cells (LBW, n = 23; NBW, n = 15) were first cultured in growth medium in 5% CO$_2$, 37°C environment, until they reached 80% confluence, designated day 0. To induce differentiation, muscle precursor cells were then cultured for 2 days in DMEM containing 1 g/L glucose, 10% FBS, and 1% PS to allow cell alignment. After 2 days, the medium was changed to DMEM containing 4.5 g/L glucose, 2% horse serum, and 1% PS. This medium was changed every 48 hours until day 7 when the cells displayed myotube morphology. At this time point, cells were visually checked for multiple nuclei to ensure differentiation. Cells were harvested at days 0, 2, 5, and 7 of the differentiation protocol. At day 7, myotubes were stimulated with 100 nM insulin, and a 2-deoxy-D-[3H] glucose uptake assay was performed (on a subgroup of cell cultures established from donors that underwent a 3-hour euglycemic-hyperinsulinemic clamp; LBW, n = 20; NBW, n = 10), and protein was harvested (LBW, n = 23; NBW, n = 15). See Supplemental Data for a detailed description.

**Western blotting**

Ten micrograms of whole cell lysates were used to detect the proteins of interest. The following antibodies were used: pAktSer473 (catalog no. 9275), Akt (catalog no. 9272), pAS160Thr442 (catalog no. 4288), pGSK3βSer9 (catalog no. 9323), and GSK3β (catalog no. 9315), all from Cell Signaling Technology; and anti-Pi3 kinase p85 (catalog no. 06–195) and GLUT1 (catalog no. 07–1401), both from Merck Millipore; citrate synthase (CS) (catalog no. ab96600; Abcam); AS160 (catalog no. 30554; Upstate Cell Signaling Solutions); MitoBiogenesis Western Blot Cocktail (catalog no. ab1235445; Abcam); GLUT4 (catalog no. PA1–1065; Thermo Scientific); and GLUT1 (catalog no. 07–1401), both from Merck Millipore. Western blotting was performed using Kaluza software version 1.2 (Beckman Coulter).

**Statistical analysis**

All analyses were performed using SAS software version 9.1.3 (SAS Institute Inc.). Non-normally distributed data were logarithmically transformed before analysis. Nontransformed data are used in the graphs. For comparisons between two groups Student’s t tests were used. To evaluate the group effect and the effects of time or insulin, an ANOVA for repeated measures was performed, and an interaction was included. The residuals obtained from the ANOVA models were evaluated, and the model was only accepted if the residuals were normally distributed. Spearman’s correlation test was used to analyze the association between DNA methylation and gene expression. Data are ex-

**Table 1. Study Group Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>LBW</th>
<th>NBW</th>
<th>P Value</th>
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<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>15</td>
<td></td>
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<tr>
<td>Birth weight, kg</td>
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<td>Height, cm</td>
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<td>Euglycemic-hyperinsulinemic clamp (M-value), mg/kg FFM/min$^a$</td>
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<td>NS</td>
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</table>

**Abbreviations:** FFM, fat free mass; HbA1C, hemoglobin A1C; NS, not significant. Data are shown as mean ± SD.

$^a$ Subgroup NBW = 10, and LBW = 20.
pressed in tables as means ± SD and in figures as means ± SEM. 

\( P < .05 \) was considered statistically significant.

**Results**

**Study group characteristics**

Individuals born with LBW had lower birth weight, current weight, height, and lean body mass compared to the NBW individuals (Table 1) and were similar in regard to lean body mass percentage, fat mass, body mass index (BMI), aerobic capacity, insulin sensitivity (M value), fasting insulin levels, fasting glucose levels, and hemoglobin A1C. A small but significant difference was observed for current age, where the NBW individuals were 1 year older than the LBW individuals.

**Purity of myogenic cultures**

To determine the effect of developmental programming on muscle stem cell function, we first isolated muscle stem cells from muscle biopsies of NBW and LBW individuals. To evaluate the composition of the isolated cells in the culture from the two birth weight groups, we assessed their cell surface marker expression by flow cytometry analysis. To discriminate myogenic cells from nonmyogenic cells, we used CD56, a positive marker of myogenic cells, and CD31 and CD45, which are expressed on endothelial and hematopoietic cells, respectively (30). In this study, all cell cultures were negative for CD31 and CD45. All cells, except one cell culture from the NBW group, were positive for CD56. This cell culture was excluded from the study (16.7% of the cells lacked CD56 expression) (Figure 1, A–C). Hence, we concluded that the cell cultures included in this study are indeed purified muscle stem cells.

**Myogenic markers**

To investigate myogenic lineage commitment and early differentiation status of the cells, Pax7 and MyoD gene

![Figure 1](https://academic.oup.com/jcem/article-abstract/101/5/2254/2804851)
expression was measured. We found that both groups had decreased Pax7 and MyoD mRNA expression throughout differentiation (Figure 2, B and C). No significant birth weight effect was found for Pax7 or MyoD gene expression (Figure 2, B and C). Myogenin mRNA expression was up-regulated over time, but was lower overall in the LBW group throughout differentiation (Figure 2D). To validate the lower myogenin gene expression in the LBW myoblasts, we measured myogenin protein level in day 7 myotubes. We found a tendency toward decreased myogenin protein levels in LBW myotubes (P = .0714) (Figure 2G).

The differentiation program is completed with the activation of muscle-specific structural proteins, such as MHC. MHC2 and MHC1 were both up-regulated at day 5 and day 7 in both groups (Figure 2, E and F), but with significantly lower MHC2 and MHC1 gene expression in myoblast from LBW compared to NBW individuals (Figure 2, E and F). In addition, we measured the number of days before the cells reached 80% confluence, designated day 0, which did not differ between groups (NBW, 11.3 ± 1.9 vs 11.4 ± 2.0 days; P = .8).

Glucose uptake, GLUT gene expression, and insulin signaling pathway

We estimated glucose uptake in myotubes in the presence or absence of insulin (100 nm for 30 minutes). Myotubes from LBW individuals showed an overall lower glucose uptake compared to those from NBW individuals, at both basal and insulin stimulated states (P < .05) (Figure 3A). We observed no significant effect of insulin on glucose uptake in any of the groups individually. Because glucose uptake is facilitated by GLUTs, we measured mRNA and protein levels in the insulin-dependent GLUT4 and the insulin-independent GLUT1. GLUT4 mRNA expression increased during differentiation of myoblasts into myotubes in both birth weight groups, but to a lower extent in cultured cells from LBW compared to NBW individuals (Figure 3B). GLUT1 mRNA expression decreased during differentiation, and the expression levels were also lower in the LBW group (Figure 3D). The mean GLUT4 and GLUT1 protein levels were lower in the LBW subjects, but the differences failed to reach statistical significance (GLUT4, P = .2225; GLUT1, P = .2653) (Figure 3G).

Figure 2. Myogenic differentiation markers. A, Myoblasts isolated from NBW (n = 15) and LBW (n = 23) individuals were grown in cell culture until mature myotubes were formed on day 7. B—F, mRNA expression of selected myogenic markers. G, Myogenin protein levels at day 7. The effect of time and birth weight was evaluated by two-way ANOVA. Data are shown as means ± SEM.
Figure 3. Glucose uptake, GLUT expression, and protein and phosphorylation status in response to insulin. A, Representative picture of day 7 myotubes from each birth weight group. Basal and insulin-stimulated (100 nM) glucose uptake was assessed by a 2-deoxy-D-[^3H] glucose uptake assay in mature myotubes (day 7) on a subgroup (NBW, n = 15; LBW, n = 23) of individuals on which a 3-hour euglycemic-hyperinsulinemic clamp (80 mIU/min) had been performed. B, GLUT4 mRNA expression. C, GLUT4 day 7 protein levels. D, GLUT1 mRNA expression. E, GLUT1 day 7 protein levels. F, Basal p85 protein level. G, Basal and insulin-stimulated pAkt^T308/total Akt protein levels. H, pGSK3β/total GSK3β protein levels. I, pAS160/total AS160 protein levels. J, AS160 mRNA expression. Performed on NBW (n = 15) and LBW (n = 23) cell cultures. Effect of time, insulin, and birth weight was evaluated by two-way ANOVA. Data are shown as means ± SEM.
To evaluate insulin signaling, myotubes were stimulated with 100 nM insulin for 30 minutes. Protein levels of the regulatory subunit (p85) of PI3 kinase as well as phosphorylation of Akt and its downstream targets glycogen synthase kinase-3β (GSK3β) and Akt substrate of 160 kDa (AS160) were measured. Basal p85 protein levels did not differ between the two groups (Figure 3F). Phosphorylation of AktSer308 was increased to a similar extent in both groups after insulin stimulation, but did not differ (Figure 3G). We found no effect of either insulin or birth weight on pGSK3βSer9 (Figure 3H). Insulin stimulation did not increase pAS160Thr642, but we found significantly lower levels in the LBW group (Figure 3I). Subsequently, AS160 levels were validated on gene expression levels during differentiation, which also showed a significantly lower AS160 gene expression in the LBW group (Figure 3J). No difference in total protein levels of Akt, GSK3β, and AS160 was found (data not shown).

Mitochondrial biogenesis

mRNA expression of PGC-1α during differentiation and nuclear encoded genes involved in OXPHOS (NDUFB6, UQCRB, COX7A, and ATP5O) were measured in the fully mature myotubes at day 7. Interestingly, we found lower levels of PGC-1α mRNA in the LBW group compared to the NBW group throughout differentiation (Figure 4A). No significant difference between groups was found for the genes NDUFB6, UQCRB, and ATP5O. However, COX7A was significantly down-regulated (P < .05) in myotubes from the LBW group (Figure 4B). As a marker of mitochondrial content and mitochondrial biogenesis, protein levels of CS, COX1, and SDH-A were measured at day 7, which did not differ between the groups (Figure 4, C and D).

DNA methylation of GLUT4, MHC2, and, PGC-1α

To investigate whether the decreased gene expression levels in the LBW group could be driven by epigenetic changes, site-specific DNA methylation was investigated in the promoters of MHC2 and PGC-1α and in the gene body of GLUT4 (Figure 5A) throughout differentiation. The specific CpG sites of PGC-1α and GLUT4 were selected based on earlier studies, which found associations between DNA methylation and fat distribution, BMI, fasting plasma insulin, or insulin sensitivity (18, 31, 32). Mean MHC2 promoter methylation was lower in the LBW group compared to the NBW group through differentiation (Figure 5B). Mean PGC-1α promoter methylation increased similarly in both NBW and LBW cells over time (Figure 5C). No effect of differentiation or birth weight was found for GLUT4 methylation (Figure 5D). We found no differences between the two groups in site-specific CpG

![Figure 4](https://academic.oup.com/jcem/article-abstract/101/5/2254/2804851)

**Figure 4.** PGC1-α and OXPHOS gene expression. A, PGC-1α mRNA expression (NBW, n = 15; LBW, n = 23) during differentiation. B, Selected OXPHOS gene expression; NDUFB6, UQCRB, COX7A, and ATP5O were measured in mature myotubes. C, CS, 70-kDa subunit of Complex II (SDH-A) and subunit I of Complex IV (COX1) protein levels measured in mature myotubes. D, Representative Western blots of CS, SDH-A, and COX1. The effect of time and birth weight was evaluated by two-way ANOVA. Student t-tests were used to investigate birth weight differences. Data are shown as means ± SEM. *, P < .05.
methylation for each gene when analyzed at individual timepoints. We only found one significant difference for MHC2 when a post hoc t test was used to look at the average CpG methylation at the different timepoints (Figure 5, B–D). We found no correlation between mean methylation levels and gene expression at the four different time-points for any of the three genes (data not shown).

Discussion

In this study, we report that LBW myotubes had decreased glucose uptake, GLUT4 and GLUT1 gene expression, as well as pAS160 protein and AS160 gene expression. LBW myotubes also displayed reduced PGC-1α gene expression, combined with decreased expression of the OXPHOS gene COX7A. Furthermore, myogenin gene expression and the muscle structural gene, MHC2 and MHC1, were down-regulated in differentiated LBW myotubes, suggesting a decreased differentiation capacity. We found a decreased average MHC2 promoter DNA methylation level in the LBW group, but no difference in DNA methylation of specific CpG sites of MHC2, PGC-1α, or GLUT4 was found between groups. Altogether, these findings show that myoblasts isolated from LBW individuals display several transcriptional and metabolic differences compared to myoblasts from NBW individuals, which seem not to be associated with birth weight-dependent alterations in methylation degrees.

In fetal life, skeletal muscle growth relies on the proliferation and differentiation of muscle progenitor cells, emphasizing the importance of correct intrauterine programming and intact intrinsic functions of the cells. Alterations in the transcriptional profile of satellite cells could potentially alter their differentiation potential and function, eventually resulting in alterations of the adult muscle tissue functions. We initially investigated the composition of isolated cells by flow cytometry analyses of cell surface markers. All cells, except from one culture that was subsequently excluded, expressed CD56, thereby confirming their myogenic potential. There were no differences in the cell marker expression between the two birth weight groups, thereby assuring us that the cultures in-

Figure 5. Selected gene methylation. DNA methylation was analyzed using pyrosequencing during myoblast differentiation (NBW, n = 15; LBW, n = 23). A, CpG sites analyzed in the GLUT4 gene body, MHC2 promoter, and PGC-1α promoter. B—D, Mean DNA methylation of selected CpG sites in GLUT4, MHC2, and PGC-1α. Data are shown as means ± SEM.
clude in this study have the same starting cell population. Our significant findings of differences in later myogenic differentiation between LBW and NBW myotubes can therefore not be explained by a difference in cell composition. We found no difference between the groups in any of the early myogenic markers, eg, Pax7 and MyoD. Both markers were down-regulated during differentiation in the two birth weight groups. Late myogenic differentiation is marked by the onset of myogenin expression, which was up-regulated in both groups during differentiation, altogether documenting that the myoblasts from both groups entered the differentiation program and developed into myotubes. However, gene expression of myogenin and MHC2 and MHC1, markers of late differentiation, were down-regulated in the LBW group, indicating a decreased or delayed ability to form myotubes. Interestingly, altered muscle fiber composition (decreased type IIa fibers) and fiber size have previously been shown in skeletal muscle biopsies from young adult LBW individuals (6). Alterations in skeletal muscle morphology and fiber composition could play a role in the pathogenesis of later development of insulin resistance and T2D in LBW individuals.

Decreased glucose uptake has been found in cultured myotubes obtained from people with T2D (19, 20). Interestingly, glucose uptake was also reduced in LBW myotubes, suggesting the presence of a prediabetic phenotype. The overall limited general response in glucose uptake to insulin is in accordance with other previous studies of similar cell lines and could be explained by a large intragroup variation in this cell cohort (19, 33). The lack of a statistically significant insulin response for glucose uptake in the two groups might also be explained by insufficient phosphorylation response on the downstream targets of Akt, eg, AS160 and GSK3β. Phosphorylation of Akt was, however, in both groups 2.5-fold higher, indicating at least some in vitro response to insulin. To further investigate the cause of decreased glucose uptake in LBW myotubes, we measured GLUT gene expression. We found decreased GLUT4 and GLUT1 mRNA expression in the cultured LBW cells during differentiation. In contrast to the mRNA levels, the trend toward reduced protein levels of GLUT1 and GLUT4 in LBW myotubes did not reach statistical significance. This may be explained by a large assay variation of the protein measurements and/or be due to differences in turnover rates of mRNA vs protein levels of these GLUTs. These small alterations in GLUTs could potentially contribute to the decreased glucose uptake, but other proteins and their phosphorylation status, including proteins like Ras-related C3 botulinum toxin substrate 1 and TUG involved in the non-Akt signaling pathway may explain the lower glucose uptake in LBW myotubes. The combined findings of decreased GLUT4, myogenin, and MHC2 and MHC1 mRNA expression indicate impaired myogenic potential in the LBW group because GLUT4 is also a marker of end-stage differentiation.

A meta-analysis study reported that LBW is associated with higher insulin resistance (34), but at the time of this study, the young LBW men were normoglycemic and did not differ significantly in their insulin sensitivity (M value) (Table 1), indicating that the changes we observe in the skeletal muscle stem cells are not a consequence of metabolic dysfunction but are likely to contribute to later development of insulin resistance and T2D. The molecular defects demonstrated in this study in cultured muscle satellite cells are indeed consistent with previous findings from intact skeletal muscle of LBW individuals (2, 5–7, 27, 35, 36). This underlines the important features of the LBW phenotype are conserved in myotubes established from LBW individuals and that the defects found in vivo in muscle may be due to intrinsic myogenic changes and dysfunctions possibly programmed early in life.

Nuclear encoded OXPHOS genes, including their key transcriptional regulator PGC-1α, are found to be down-regulated in skeletal muscle of T2D patients and LBW individuals after 5 days of high-fat overfeeding (7, 25, 37, 38). In this study, we found reduced COX7A and PGC-1α gene expression in LBW myotubes, again indicating a conserved phenotype during in vitro muscle satellite cell differentiation (7). However, we found no difference in selected mitochondrial protein levels of CS, COX1, and SDH-A. A previous study reported an inverse association between PGC-1α gene expression and promoter DNA methylation in T2D patients and controls (39). Interestingly, in two previous cohorts of mature skeletal muscle tissue from LBW individuals and matched controls, we found increased PGC-1α promoter methylation in LBW subjects studying the same promoter region, but no difference in PGC-1α gene expression (7, 28). Here, we found no effect of birth weight on PGC-1α methylation levels and no significant correlation between the PGC-1α promoter methylation degree and gene expression. Together, this could question the role of PGC-1α DNA methylation as a key regulator of PGC-1α expression in muscle stem cells at prediabetic states. We suggest that PGC-1α gene expression is regulated by other mechanisms in the myotubes, eg, posttranslational mechanisms, histone modifications, microRNAs, or alternatively regulated by DNA methylation at other CpG or non-CpG methylation sites (39).

Mean MHC2 promoter methylation and gene expression were both lower in the LBW group compared to the NBW group, suggesting that DNA methylation at the investigated CpG sites in the MHC2 promoter is not nega-
tively associated with MHC2 gene expression. Furthermore, average methylation percentage of the MHC2 CpG sites was medium to high (50–55%), indicating that this region in the promoter is not crucial for transcription factor binding (40). Alternatively, the MHC2 gene expression may be regulated by distal elements (eg, enhancers), histone modifications, or DNA methylation at other CpG sites in the promoter of MHC2. However, because the mean MHC2 promoter methylation was significantly lower in LBW compared to NBW individuals, it is possible that CpG methylation at the sites investigated in our study somehow reflects the fetal development.

In this study, we demonstrate several alterations in cultured primary myotubes derived from LBW individuals compared to myotubes derived from NBW individuals. The fact that the LBW myotubes have retained their underlying programmed phenotype when cultured during standardized conditions in vitro, and even after multiple cell divisions, demonstrates that the previously documented defects in muscle from LBW subjects (2–7) may not be secondary to other metabolic perturbations in vivo, including mild hyperglycemia and hyperinsulinemia, but may be primary and intrinsically preprogrammed defects.

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Author Contributions: A.V. 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. N.S.H., L.H., C.B., and A.V. designed the cell study. B.M., S.W.J., J.F.P.W., and A.V. designed the human cohort study. N.S.H., L.H., C.B., L.G., M.S., B.M., H.S.S., and S.W.J. performed the experiments. N.S.H., C.B., L.G., H.S.S., and A.V. analyzed the data. N.S.H., C.B., L.G., and A.V. interpreted the results of the experiments. N.S.H. prepared the figures. N.S.H., L.H., C.B., and A.V. drafted the manuscript. N.S.H., L.H., C.B., L.G., M.S., B.M., S.W.J., M.F., J.F.P.W., B.K.P., and A.V. edited and revised the manuscript. All authors approved the final version of the manuscript. A.V. and B.K.P. are members of DD2, the Danish Center for Strategic Research in Type 2 Diabetes (the Danish Council for Strategic Research, Grants 09–067009 and 09–075724). B.K.P. and J.F.P.W. are partners of the UNIK Project: Food, Fitness & Pharma for Health and Disease, supported by the Danish Ministry of Science, Technology, and Innovation.


References


