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ORIGINAL PAPER

# Maternal protein restriction induce skeletal muscle changes without altering the MRFs MyoD and myogenin expression in offspring

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**Abstract** Stimuli during pregnancy, such as protein restriction, can affect morphophysiological parameters in the offspring with consequences in adulthood. The phenomenon known as fetal programming can cause short- and long-term changes in the skeletal muscle phenotype. We investigated the morphology and the myogenic regulatory factors (MRFs) MyoD and myogenin expression in soleus, SOL; oxidative and slow twitching and in extensor digitorum longus, EDL; glycolytic and fast twitching muscles in the offspring of dams subjected to protein restriction during pregnancy. Four groups of male Wistar offspring rats were studied. Offspring from dams fed a low-protein diet (6 % protein, LP) and normal protein diet (17 % protein, NP) were euthanized at 30 and 112 days old, and their muscles were removed and kept at −80 °C. Muscles histological sections (8 μm) were submitted to a myofibrillar adenosine triphosphatase histochemistry reaction for morphometric analysis. Gene and protein expression levels of MyoD and myogenin were determined by RT-qPCR and western blotting. The major findings observed were distinct patterns of morphological changes in SOL and EDL muscles in LP offspring at 30 and 112 days old without changes in MRFs MyoD and myogenin expression. Our results indicate that maternal protein restriction followed by normal diet after birth induced morphological changes in muscles with distinct morphofunctional characteristics over the long term, but did not alter the MRFs MyoD and myogenin expression. Further studies are necessary to better understand the mechanisms underlying the maternal protein restriction response on skeletal muscle.

**Keywords** Skeletal muscle · Maternal protein restriction · Pregnancy · Fetal programming · Myogenic regulatory factors

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## Introduction

Skeletal muscle is a highly plastic tissue that adapts to changes in nutritional status by changing its size, metabolism and functional properties. This tissue also could be affected in offspring in response to maternal undernutrition, changing the phenotype in adulthood (Fahey et al. 2005; Zhu et al. 2006; Mallinson et al. 2007; Toscano et al. 2008). Nutritional interventions during pregnancy determine

a consistent series of disorders in offspring (Barker et al. 1994; Langley-Evans et al. 2004; Mesquita et al. 2010) in a process known as "fetal programming" (Lucas 1991). Recently, many studies have demonstrated a link between fetal programming and many diseases, such as cardiovascular disorders, hypertension, osteoporosis, obesity and insulin resistance, over a long period of time (Fowden et al. 2006; Kevin and Sinclair 2007). Insulin resistance can alter the metabolic and functional capacity of skeletal muscle (Phillips 1994; Ozanne et al. 2003).

During muscle development, the physiological and biochemical properties of muscle fibers are determinate. The first fibers formed are defined as primary fibers, with secondary fibers forming slightly later, around the primary. In general, the primary fibers tend to become type I (slow), while the secondary tend to become type II (fast) fibres (Wilson et al. 1988). In muscles of adult rats, based on myofibrillar actomyosin adenosine triphosphatase (mATPase) histochemistry, four pure fibers are described: Type I, IIA, IID and IIB. Thse muscle fibers express four pure myosin heavy chain (MHC) isoforms: slow, MHCI and three fast, MHCIIa, MHCIId and MHCIIb, respectively (Staron et al. 1999). In addition, hybrid muscle fibers, IC, IIC, IIAD and IIDB presenting two MHC isoforms in combinations (MHCI + MHCIIa, MHCIIa + MHCIId, or MHCIId + MHCIIb), respectively also are described. These "hybrid" fibers suggests a continuous of fiber types spanning from the slowest (type I) to the fastest (type IIB) by "bridging the gap" between the pure fiber types I, IIA, IID, and IIB: I ↔ IC ↔ IIC ↔ IIA ↔ IIAD ↔ IID ↔ IIDB ↔ IIB (Staron et al. 1999). Postural muscles, as SOL are constituted predominantly by type I (oxidative and slow twitching muscle) fibers and fast phasic muscles, as EDL (glycolytic and fast twitching muscle) are formed predominantly by type II muscle fibers.

During fetal development, skeletal muscle can be altered by stimuli, such as nutrient availability (Bedy et al. 1982; Beermann 1983; Wilson et al.1988; Ozanne et al. 2003; Mallinson et al. 2007). In fact, undernutrition during critical periods of development can influence muscle fiber formation and development in the offspring by the down-regulation of genes related to the muscle growth process (Bayol et al. 2004).

Studies that examine the effects of undernutrition during the early stages of muscle development in offspring normally include the pregnancy and lactating stages, and it is clear that undernutrition before weaning causes permanent effects in skeletal muscle over the long term (Bedy et al.1982; Wilson et al. 1988; Beermann 1983; Ozanne et al. 2003). However, undernutrition during pregnancy stages, excluding the lactating phase, has shown variable responses in the skeletal muscle phenotype in different species that are related to the type of restrictions in the diet and the gestational period (Fahey et al. 2005; Zhu et al. 2006; Mallinson et al. 2007; Daniel et al. 2007; Toscano et al. 2008; Huber et al. 2009).

Different pathways regulate skeletal muscle phenotypic changes (Loughna and Brownson 1996; Bayol et al. 2004; Sandri 2008), including the myogenic regulatory factors (MRFs), which comprise a family of transcriptional factors that control the expression of skeletal muscle-specific genes (Hughes et al. 1993). The family has four members: MyoD, myogenin, Myf5 and MRF4. MRFs form dimers with the ubiquitous E proteins (e.g., E12 or E47), which results in heterodimeric complexes that bind to the E-box consensus DNA sequence (50-CANNTG-30) that is found in the regulatory region of many muscle-specific genes (Murre et al. 1989). The MRFs are critical for establishing the myogenic lineage and for the terminal differentiation of myoblasts (Parker et al. 2003). Studies have suggested that MyoD and myogenin may also be involved in establishing and maintaining of slow and fast mature muscle fibre phenotype; myogenin is expressed at higher levels compared with MyoD in slow muscles, whereas the opposite is true for fast muscles (Lin et al. 1991; Hughes et al. 1993; Voytik et al. 1993; Allen et al. 2001; Alway et al. 2001). Thus, we can suggest that the MRFs MyoD and myogenin specifically contribute controlling skeletal muscle phenotype.

Recent research has led to advances in our understanding of the cellular and molecular events involved in tissue adaptation during fetal programming. Because little is known about the factors involved in the skeletal muscle changes that occur in offspring subjected to maternal protein restriction, we investigated the morphological characteristics and the MRFs MyoD an myogenin expression levels in soleus, SOL; oxidative and slow twitching and in extensor digitorum longus, EDL; glycolytic and fast twitching muscles in male offspring of dams fed a low-protein diet during all of the gestational phases.

## Materials and methods

### Experimental model

Our experiment was performed in accordance with the Biosciences Institute Ethics Committee, UNESP, Botucatu, SP, Brazil (Protocol No. 081/07-CEEA). The animals had ad libitum access to food and water at all times and were housed in temperature-controlled conditions on a 12 h light–dark cycle. Virgin female Wistar rats with a body weight between 250 and 300 g were housed individually with males under standardized conditions. After mating, pregnancy was determined by the examination of vaginal smears to detect the sperm. Sixteen pregnant rats were

**Table 1** Composition of the experimental diets

| Ingredient (g/kg) | Normal-protein diet (17 % protein) | Low-protein diet (6 % protein) |
|---|---|---|
| Cornstarch | 397 | 480 |
| Dextrin | 130.5 | 159 |
| Saccharose | 100 | 121 |
| L-Cystine | 3 | 1 |
| Fiber | 50 | 50 |
| Soybean oil | 70 | 70 |
| Mineral mix AIN93 G[a] | 35 | 35 |
| Vitamin mix AIN93 G[b] | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 |

[a] American Institute of Nutrition 93; mineral mix provided (mg/kg diet): calcium 5.0, phosphorus 1.6, sodium 1.0, potassium 2.3, magnesium 0.5, iron 0.03, zinc 0.03, and copper 0.01

[b] American Institute of Nutrition 93G; vitamin mix provided (mg/kg diet): nicotinic acid 30.0, calcium pantothenate 16.0, pyridoxine-HCl 7.0, thiamin-HCl 6.0, riboflavin 6.0, folic acid 2.0, biotin 2.0, cyanocobalamin, 25.0, -tocopherol 150.0, retinyl palmitate 8.0, cholecalciferol 2.5, and phylloquinone 0.75. (Reeves 1993)

isolated and randomly divided into two experimental groups: a control group of dams fed a normal protein diet (17 % protein, NP, N = 8) and group of dams fed low-protein diet (6.0 % protein, LP,N = 8) (Table 1). The diets were isocaloric and were administered from first day until the end of the pregnancy. The diets were produced in Physiology Laboratory of University of Campinas, Brazil (UNICAMP) as per (Reeves 1993) in according with American Institute of Nutrition to AIN-93 recommendations. From the birth of the offspring (day 21), the dams had ad libitum access to Purina standard rat chow (Labina, Brazil), which contained (w/w) 57.5 % starch, 23 % protein, 2.5 % lipid, 9 % cellulose, 8 % vitamins and salts in according with National Research Council National Institute of Health, USA, recommendations, until offspring weaning.

At birth, the litter sizes were standardized to eight randomly chosen pups (four males and four females) in each litter. The female pups were not used in the analysis but were maintained until weaning to maintain equal availability of food between offspring. After weaning, all of the male rats were fed ad libitum with standard rat chow (Purina Labina, Brazil), until 112 days old. We studied four groups: the $NP^{30d}$ group (n = 8) included the 30-day-old offspring of dams fed with a normal protein diet (17 % protein) during the entire pregnancy; the $LP^{30d}$ group (n = 8) included the 30-day-old offspring of dams fed a low-protein diet (6 % protein) during the entire pregnancy; the $NP^{112d}$ group (n = 8) included the 112-day-old offspring of dams fed a normal protein diet (17 % protein) during the entire pregnancy; and the $LP^{112d}$ group (n = 8) included the 112-day-old offspring of dams fed a low-protein diet (6 % protein) during the entire pregnancy. The aim of our study was to evaluate the effect of maternal protein restriction in the muscles of young and adult rats, considering 30 and 112-days-age as the endpoint.

At each endpoint, the rats were anesthetized with an intraperitoneal injection of a 2:1 solution of ketamine hydrochloride (Ketamine®) at 50 mg/mL and xylazine hydrochloride (Xylazine®) at 20 mg/mL at a dose of (0.1 mL/100 g) and were euthanized. The extensor digitorum longus (EDL) and soleus (SOL) muscles were collected, frozen in liquid nitrogen at −156 °C and stored at −80 °C.

## Histochemical and morphometric analysis

Histological sections (8 μm) from the mid-belly portion of the EDL and SOL muscles were obtained in a cryostat (JUNG CM1800, Leica, Germany) at −24 °C. The muscle fiber-type frequency (%) and the cross-sectional area (CSA) were determined using myofibrillar adenosine triphosphatase (mATPase) histochemistry with preincubation in a pH of 4.3 for EDL muscle and in a pH of 4.4 for SOL muscle. The EDL muscle exhibited the pure muscle fibers I, IIA, IID and IIB. Muscle fibers type IIA and IID were grouped (IIA + IID). The SOL muscle presented pure (I and IIA) and hybrid (IC and IIC) muscle fibers (Staron et al. 1999). Muscle fibers type IC and IIC were grouped (IC + IIC) .

The muscle fiber-type frequency and CSA were determined using Image Analysis System Software (LeicaQWin Plus, Germany) coupled with a Leica DM. The counts were performed on transverse sections of each muscle, and the fiber-type frequency and area were determined by counting approximately 200 fibers per animal equally distributed over the muscle sample.

## Gene expression analysis

### *RNA isolation and analysis*

The gene expression of MRFs was assessed by real-time PCR. Total RNA was extracted from the muscle samples with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate method. The frozen muscles were mechanically homogenized in 1 mL of TRIzol Reagent. Total RNA was resuspended in RNase-free water and treated with DNase I (Life Technologies, Carlsbad, CA, USA) to remove any possible DNA present in the sample. The total RNA was quantified using a Nanodrop spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) to measure the optical density (OD) at 260 nm. RNA purity was ensured when the $OD_{260/280}$ ratio was approximately 2.0. Additionally, RNA

integrity was verified by 1 % agarose gel electrophoresis, in which well-defined bands were observed corresponding to the 18S and 28S ribosomal RNAs.

*Reverse transcription*

For each sample, cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit (Life Technologies, Carlsbad, CA, USA). Each reaction contained 10 μL of 10× reverse transcription buffer, 4 μL of 25× dNTPs, 10 μL of 10× random primers, 100 units of RNase inhibitor (Life Technologies, Carlsbad, CA, USA) and 250 units of Reverse Transcriptase MultiScribe™, and the final volume was adjusted to 200 μL with nuclease-free water. The cDNA synthesis reaction conditions were 10 min at 25 °C for primer annealing followed by 2 h at 37 °C for reverse transcription. A control reaction was performed by omitting the reverse transcriptase enzyme. All of the cDNA samples were amplified by PCR to ensure that there was no contamination by DNA. The resulting cDNA samples were aliquoted and stored at −20 °C. A volume of 2 μL of cDNA, which corresponds to 20 ng of total RNA, was used in real-time PCR reactions.

*Real-time qPCR*

The cDNA of each sample was used as the template in real-time PCR reactions, which were performed in a 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with universal thermal cycling conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were performed in duplicate using 0.4 μM of each primer and 2× Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) in a final volume of 25 μL. Gene sequences were selected from the accession numbers in the National Center for Biotechnology Information database (Table 2). Primer sequences were designed using the primer design function of the software Primer Express v3.0 (Life Technologies, Carlsbad, CA, USA). For each gene, a series of five dilutions (10×) was performed on PCR products that were diluted 500 times from an initial mixture containing equal amounts of cDNA from all samples, which allowed the generation a five-point standard curve for each primer set initially chosen for qPCR. The qPCR linearity and efficiency were calculated using the slope of the standard curve, which was generated using 7300 System SDS software (Life Technologies, Carlsbad, CA, USA) for each gene analyzed. The analysis of all of the standard curves showed high linearity ($r^2 = 0.99$). The PCR efficiency (Ex) was calculated from the equation $Ex = 10 - 1^{-1/slope} - 1$. A slope of −3.32 implies a reaction efficiency of 100 %. All of the genes had a slope of approximately −3.32, and the estimates of efficiency were between 98.0 and 100.5 %. Gene expression was compared between individual samples using the ΔΔCq method described by Livak and Schmittgen (2001). It was also necessary, prior to the final quantification, to perform a correction for experimental variability between different samples, for example, for the amount of RNA and the

**Table 2** Primers used for real-time PCR amplification of reverse transcribed RNA product

| Product | Accession no | Sequence (50–30) |
|---|---|---|
| MyoD | NM_176079 | F: TTTTTCATGCGACTCACAGC |
| | | R: GAAGGCAGGGCTTAAGTGTG |
| Myogenin | M24393 | F: GTCTTTTCCGACCTGATGGA |
| | | R: ATAGAAGTGGGGCTCCTGGT |
| ARBP[a] | NM_022402 | F: CCTGCACACTCGCTTCCTAGAG |
| | | R: AACAGTCGGGTAGCCAATCTG |
| TBP[a] | NM_001004198 | F: GCCACGAACAACTGCGTTGAT |
| | | R: AGCCCAGCTTCTGCACAACTCTA |
| GAPDH[a] | AB017801.1 | F: TGCACCACCAACTGCTTA |
| | | R: GGATGCAGGGATGATGTTC |
| HPRT[a] | NM_000194.1 | F: TGACACTGGCAAAACAATGCA |
| | | R: GGTCCTTTTCACCAGCAAGCT |
| ACTB[a] | NM_031144 | F: CAGGTCATCACTATCGGCAATG |
| | | R: TTTCATGGATGCCACAGGATTC |

Accession no, GenBank accession number. F: primer senso; R: primer anti-senso. GAPDH: gliceraldeido 3-fosfato desidrogenase, TBP: TATA binding box protein, ARBP: acidic ribossomal phosphoprotein, HPRT: hypoxanthine phosphoribosyl-transferase, ACTB: actin beta. Interest genes: MyoD and myogenin

[a] Genes that were assessed. The TBP, GAPDH and ARPB were used for normalization as reference genes

reverse transcription reaction efficiency. Data normalization to at least three reference genes is the most accepted method for avoiding disparities due to experimental variability (Vandesompele et al. 2002). Choosing appropriate genes is crucial for reliable results, and the expression level of these genes must remain unchanged between the different experimental conditions (Alway et al. 2002; Sanchez et al. 2006). The expression levels of five genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine–guanine phosphoribosyltransferase (HPRT) TATA box binding protein (TBP) and acidic ribosomal phosphoprotein (ARBP), actin beta (ACTB) were assessed. Only expression levels of three genes (TBP, GAPDH and ARPB) were chosen using DataAssist software v 2.0. from Life Technologies (Carlsbad, CA, USA) and were used for normalization as reference genes. The software was also used for quantifying relative gene expression.

### Protein expression analysis

The protein levels of MyoD and myogenin in SOL and EDL muscles were determined by Western blotting using $\beta$-actin protein to normalize. The muscle samples were individually homogenized in 400–500 µL lysis buffer (1 % Triton X-100, 10 mM sodium pyrophosphate, 100 mM NaF, 10 µg/mL aprotinin, 1 mM phenylmethylsulfonyl-fluoride (PMSF), 0.25 mM $Na_3VO_4$, 150 mM NaCl, 10 mM EDTA, and 50 mM Tris–HCl pH 7.5). The samples were centrifuged at 11,000 rpm for 20 min at 4 °C for the supernatant collection. The homogenate fraction was resuspended in Laemmli loading buffer (2 % SDS, 20 % glycerol, 0.04 mg/mL bromophenol blue, 0.12 M Tris–HCl pH 6.8, and 0.28 M $\beta$-mercaptoethanol) at 95–100 °C for 5 min. Proteins were quantified using the Bradford method using bovine serum albumin (BSA 0.1 %) as protein standard. An amount of 50 µg of total protein was fractioned by one-dimensional SDS-PAGE (12 %), and the gel was stained with Coomassie blue to confirm the equal loading of each sample. Proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). Nonspecific binding sites were blocked using 5 % skim milk/Tris–HCl-buffered saline-Tween buffer [TBS-T: 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.05 % Tween-20] followed by specific primary antibody incubation overnight at 4 °C (Table 3). After three wash steps using TBS-T buffer, the membranes were incubated with specific horseradish peroxidase-conjugated secondary antibodies according to the primary antibodies used (Table 3). The procedures varied according to each specific protein analyzed in the blocking solution concentration, the blocking time period, and the primary and secondary antibody dilutions used.

Immunoreactive protein signals were detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Rockford, IL, USA) according manufacturer's recommendations. The signal was captured on immunoblotting film, and the band intensities were quantified using densitometry analysis software (Image J software for Windows, version 1.71, 2006, Austria). The values were normalized by the $\beta$-actin protein results.

### Statistical analysis

The muscle fiber cross-sectional area (CSA) and the gene and protein expression values were compared between groups (NP and LP) using Student's unpaired $t$ test. The muscle fiber frequency was analyzed using the Mann–Whitney test (Zar 2009). Significance was assumed at a level of $p < 0.05$.

## Results

### Muscle weight in offspring

At 30 days, the SOL muscle weight (g) was significantly lower in LP compared to NP group and no significant difference was observed in the EDL muscle weight (Fig. 1a). There were no significant differences in the SOL and EDL muscles weight/body weight ratio between the experimental groups in this period (Fig. 1b). At 112 days, no significant difference was observed in the SOL and EDL muscles weight between the experimental groups (Fig. 1c). However, the SOL muscle weight/body weight ratio was significantly lower in LP compared to NP group; no significant difference was observed in the EDL muscle weight/body weight ratio between the experimental groups (Fig. 1d).

**Table 3** Primary and secondary antibodies used in Western Blot analysis

| Protein | Primary antibody | Secondary antibody | Manufacturer |
|---|---|---|---|
| MyoD | M-318 (sc-760) | Goat anti-rabbit IgG-HRP/sc-2004 | Santa Cruz Biotechnology California, USA |
| Myogenin | M-225 (sc-576) | Goat anti-rabbit IgG-HRP/sc-2004 | |
| $\beta$-actin | R-22 (sc-130657) | Goat anti-rabbit IgG-HRP/sc-2004 | |

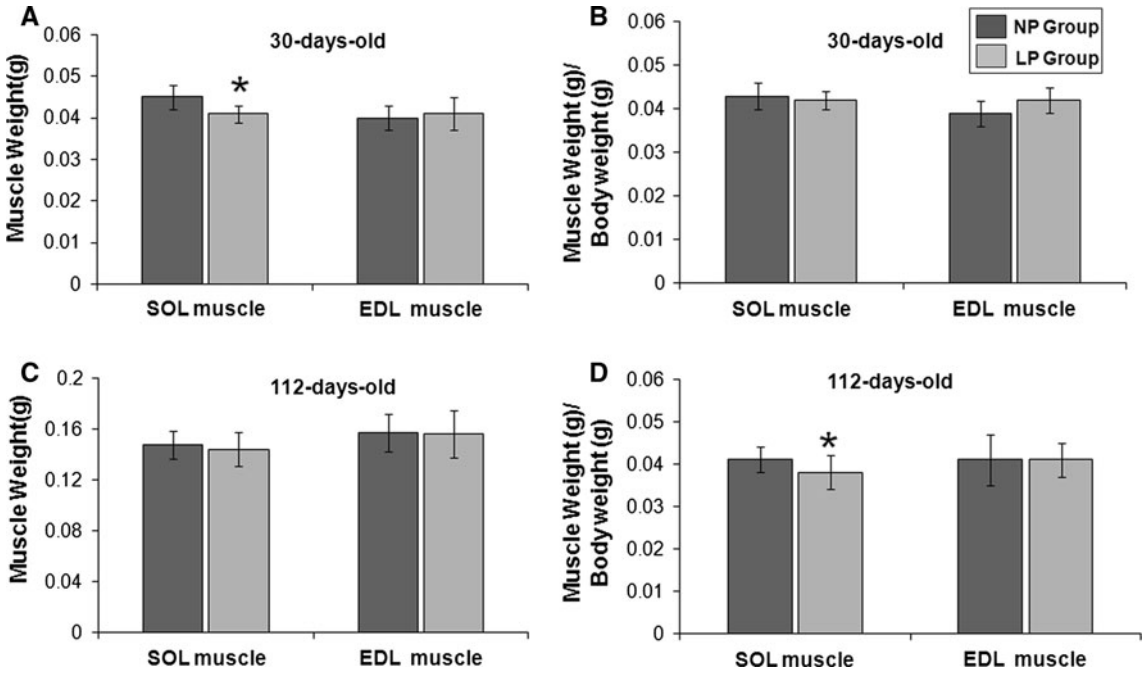


**Fig. 1** Muscle weight and muscle weight/body weight ratio of offspring. The values are expressed as the mean ±SD. *$p < 0.05$ statistically different versus the NP group

### Histochemical and morphometric analyses in offspring

At 30 days of age, no statistical difference was observed between groups in the EDL CSA in fiber types I, IIA + IID and IIB (Fig. 2a). However, the SOL muscle of LP group exhibited decreased CSA in the fiber type I in comparison to the NP group at 30 days (Fig. 2b). At 112 days old, the same pattern was observed in the CSA of the EDL (Fig. 2c) and the SOL (Fig. 2d) muscles.

With regards to muscle fiber-type frequency, at 30 days, we observed an increase in the muscle fiber frequency of type IIB and a decrease in types I and IIA + IID in the EDL muscle of the LP group compared to the NP group. No statistically significant difference in fiber frequency was observed in the SOL muscle at this time-point (Table 4). At 112 days of age, the offspring showed no changes in the fiber type frequency in the SOL or EDL muscles (Table 4).

### Analysis of MyoD and myogenin gene expression in offspring

The gene expression levels of MyoD and myogenin are shown in Table 5. At 30 and 112 days of age, no changes were observed in MyoD or myogenin gene expression in EDL and SOL muscles in the LP groups compared to the NP groups.

### Analysis of MyoD and myogenin protein expression in offspring

The MyoD and myogenin protein expression levels are shown in the Fig. 3. At 30 and 112 days of age, no difference was observed in MyoD or Myogenin protein expression in EDL and SOL muscles in the LP groups compared to the NP groups.

## Discussion

The aim of this study was to investigate the morphology and the myogenic regulatory factors (MRFs) MyoD and myogenin expression in soleus, SOL; oxidative and slow twitching and in extensor digitorum longus, EDL; glycolytic and fast twitching muscles in the offspring of dams subjected to protein restriction during pregnancy. We showed that protein restriction during pregnancy induced fiber type I hypotrophy in the SOL muscle of the offspring at 30 and 112 days of age. The EDL muscle fiber type was modulated toward a more fast muscle type only in the LP group at 30 days, and no differences were observed in MyoD and myogenin expression in SOL and EDL muscles in the LP group compared to the NP group at both ages.

Skeletal muscles are composed by fiber types that have specific metabolic and functional activities (Pette and Staron 2000; Fluck and Hoppeler 2003; Magaud et al.

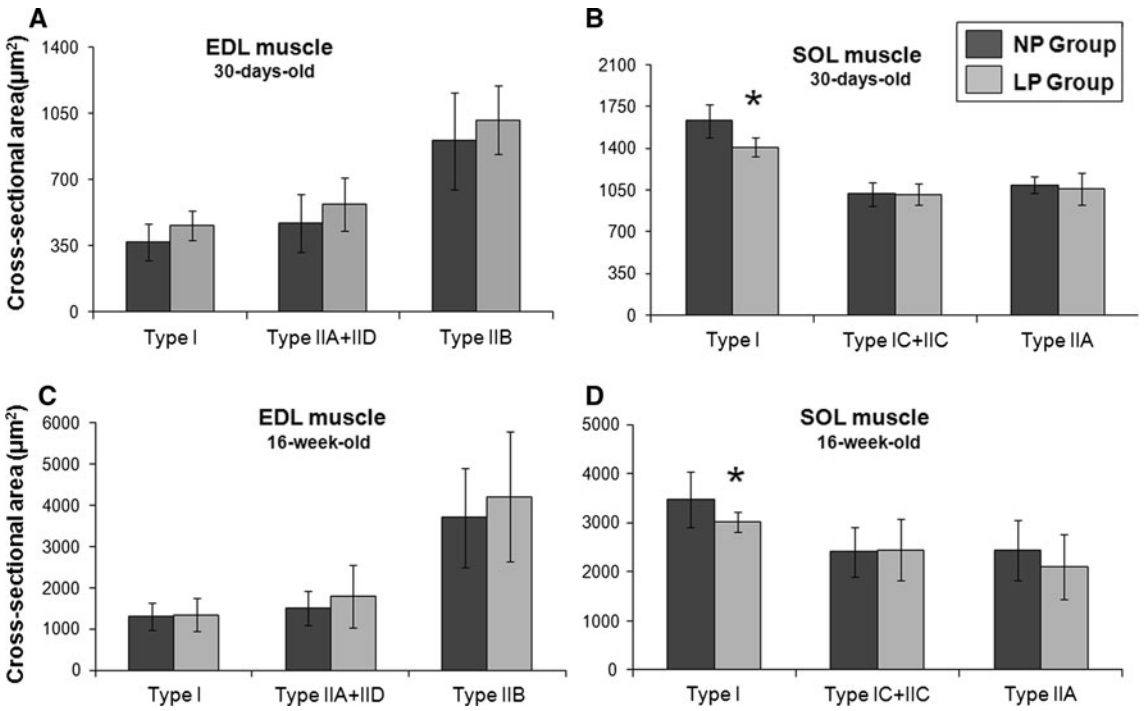


**Fig. 2** Cross sectional area (CSA) of EDL (**a**, **c**) and SOL (**b**, **d**) muscle fibers from 30 and 112-days-old offspring. NP and LP groups are represented by *black* and *gray bars*, respectively. The values are expressed as the mean ±SD. *$p < 0.05$ statistically different versus the NP group

**Table 4** Muscle fiber-type frequency

| Muscle | 30-days-old offspring | | 112-days-old offspring | |
|---|---|---|---|---|
| | NP group (n = 6) | LP group (n = 6) | NP group (n = 8) | LP group (n = 8) |
| EDL (%) | | | | |
| Type IIB | 31.4 (28.7; 45.9) | 45.1 (40.3; 59.6)* | 47.0 (38.6; 50.9) | 46.7 (37.4; 49.6) |
| Type IIA + IID | 55.0 (18.0; 61.3) | 45.8 (26.3; 50.8)* | 41.0 (35.1; 44.2) | 41.6 (37.1; 45,6) |
| Type I | 12.2 (9.2; 36.0) | 8.8 (6.7; 14.0)* | 12.8 (10.1; 18.5) | 11.8 (9.7; 23.1) |
| SOL (%) | | | | |
| Type I | 56.6 (53.1; 63.1) | 60.0 (53.5; 67.9) | 73.1 (56.7; 80.9) | 77.9 (53.3; 84.3) |
| Type IC + IIC | 41.3 (32.5; 43.6) | 42.1 (36.9; 53.1) | 18.5 (10.4; 35.0) | 16.7 (8.3; 41.7) |
| Type IIA | 23.2 (18.4; 26.7) | 22.9 (13.3; 31.1) | 8.2 (6.0; 13.6) | 5.4 (2.4; 12.2) |

Values are expressed as median (minimum–maximum value). Soleus muscle (SOL) and extensor digitorum longus muscle (EDL). Values that are significantly different from the control group (NP) are denoted by an asterisk (*)

2004). These characteristics can be changed in several conditions (Walters et al. 2000; Psilander et al. 2003; Dow et al. 2004; Jackman and Kandarian 2004; Magaudda et al. 2004), including protein energy restriction imposed during pregnancy. Protein energy restriction during pregnancy can induce changes in the muscle phenotype in adulthood (Fahey et al. 2005; Zhu et al. 2006, Mallinson et al. 2007; Toscano et al. 2008).

In our work, offspring of 30 and 112 days of age exhibited a reduced CSA of fiber type I in SOL muscle which also showed reduced weight. No change in muscle fiber-type frequency was observed in this muscle comparing the LP and NP groups at both of the time-points analyzed. According to Mallinson et al. (2007), food restriction causes changes in the muscle fiber-type frequency in SOL muscle only during specific stages of gestation. The authors observed that in 4-week-old offspring, an increase in SOL fiber type I frequency was observed when the maternal protein restriction (9 vs. 18 %) occurred only in the early stages of pregnancy (0–7 days); a reduction in SOL fiber type II frequency was observed when the restriction was imposed for an intermediate stage of pregnancy (8–14 days). When the restriction was imposed for the entire pregnancy period (0–22 days), the

**Table 5** Relative gene expression (mRNA) of MyoD and Myogenin

| Muscle | 30-days-old offspring | | 112-days-old offspring | |
|---|---|---|---|---|
| | NP group (n = 8) | LP group (n = 8) | NP group (n = 8) | LP group (n = 8) |
| EDL | | | | |
| MyoD | 0.95 ± 0.07 | 0.83 ± 0.20 | 0.79 ± 0.44 | 1.07 ± 0.56 |
| Myogenin | 0.86 ± 0.17 | 1.15 ± 1.02 | 1.10 ± 1.02 | 0.69 ± 0.21 |
| SOL | | | | |
| MyoD | 1.75 ± 1.03 | 1.38 ± 0.44 | 0.84 ± 0.72 | 1.42 ± 1.11 |
| Myogenin | 0.79 ± 0.15 | 0.69 ± 0.21 | 1.47 ± 0.32 | 1.32 ± 0.53 |

Values are expressed as means ±SD. Soleus muscle (SOL) and extensor digitorum longus muscle (EDL). No statistical difference was observed, $p > 0.05$

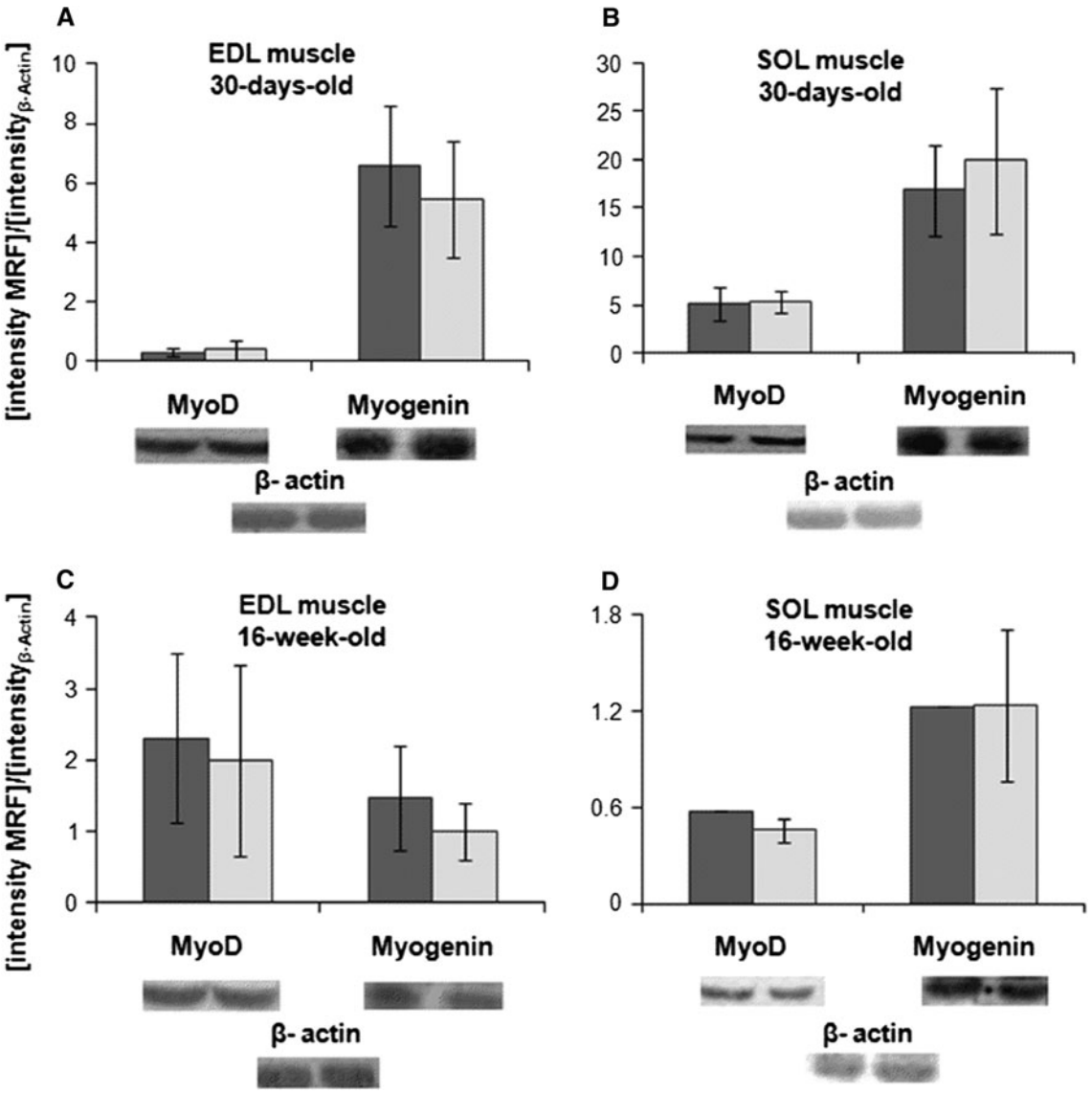


**Fig. 3** MyoD and myogenin protein expression in the EDL (**a**, **c**) and SOL (**b**, **d**) muscles, 30 (**a**, **b**) and 112-days-old (**c**, **d**) offspring. NP and LP groups are represented by *black* and *gray bars*, respectively. The values are expressed as the mean ±SD

SOL muscle fiber type frequency did not change (Mallinson et al. 2007). However, Toscano et al. (2008) observed an increase in SOL fiber type IIA frequency in offspring of 25 and 90 days of age in response to protein restriction (7.8 vs. 17 %) during the entire pregnancy period (0–21 days). Considering the restriction period used in our study (0–21 days), the SOL muscle fiber type frequency was similar to that shown by Malisson et al. (2007). The conflicting results regarding the effects of undernutrition during pregnancy can be related to diet type, the gestational period in which the diet is used and the diet type used after birth (Fahey et al. 2005; Zhu et al. 2006; Malisson et al. 2007; Daniel et al. 2007; Toscano et al. 2008; Huber et al. 2009). In our study, the protein level of the diet offered to the offspring during the postnatal period (23 %) was more similar to that used by Malisson et al. (2007, 20 %) than that reported by Toscano et al. 2008), that used protein amounts from 17 to 12 %. Therefore, the variations in these parameters could explain the differences in the results related to the SOL muscle fiber type frequency.

In our study, the decreased SOL muscle type I fiber CSA in LP group was accompanied by a reduced SOL muscle weight. SOL muscle is primarily constituted of oxidative and slow muscle fibers that are recruited for continuous and sustained activities (Johnston 1985) and require a higher rate of protein turnover (Davis et al. 1989). The protein restricted diet used during the entire pregnancy probably affected this characteristic in SOL muscle and it is possible that the diet used during the postnatal period was insufficient to recover the SOL CSA, condition that can be defined as hypotrophy (Cullen et al. 1992). Considering that at birth, the body weight of pups was statistically ($p < 0.05$) lower in LP compared to NP group ($5.7 \pm 0.6$ and $6.2 \pm 0.5$, respectively), we can infer that the diet used during pregnancy could have influenced the tissue development and growth.

Zhu et al. (2004) showed that mTOR, a factor involved in protein synthesis, was decreased in muscle of sheep offspring in response to maternal nutrient restriction. Although the mechanisms by which maternal protein-restricted diet influences the muscle CSA remain unknown, we speculate that a change in the pathway involved in protein synthesis occur during muscle hypotrophy. This adaptive change can affect the SOL muscle morphophysiology and can predispose the offspring to disease in later life.

In EDL muscle, we observed changes in fiber-type frequency only in the LP group at 30 days. There was an increase in the frequency of fiber type IIB and a decrease in that of types I and IIA + IID. This result could contribute to the pattern of EDL insulin resistance. Humans with low birth weight (LBW) in response to maternal undernutrition exhibit increased in IIX/IID, a decrease in IIA and no change in I fiber-type frequency in vastus lateralis muscle (Jensen et al. 2007). The authors correlated these results with the development of type 2 diabetes, as was also discussed by Toscano et al. (2008). In support of this conclusion, muscles with predominantly type II fibers have less capacity to respond to insulin and a low rate of glucose transporter (GLUT4) activity (Megeney et al. 1993; Daugaar et al. 2000; Halseth et al. 2001).

EDL is a fast glycolytic muscle that is recruited during non-sustained, fast activities (Johnston 1985) and has a lower protein turnover rate than SOL muscle (Davis et al. 1989). During EDL myogenesis, the primary fibers are slow and the secondary fibers are fast. The majority of primary slow fibers are changed to fast fibers (Kelly and Rubinsten 2003). It is possible that the maternal protein restriction imposed in our experiment enhanced type II muscle fiber differentiation during myogenesis (Kelly and Rubinsten 2003). At 112 days, the EDL muscle from LP group exhibited no change in the fiber-type frequency. This demonstrates that the diet used post-birth allowed the EDL muscle to recover a pattern similar to that of the NP group. EDL muscle did not exhibit hypotrophy in our study. This result is intriguing considering that SOL muscle exhibited this characteristic. However, we need to consider that these muscles have distinct metabolic and contractile characteristics (Davis et al. 1989) and myogenic processes (Kelly and Rubinsten 2003). We predict that our CSA results reflect the EDL muscle physiology. However, other experiments must be conducted to better explain these results.

In our experiment, no significant change in the MRFs Myod and myogenin expression was observed between the LP and NP groups for both ages and muscles analyzed. These transcriptional factors, together with Myf5 and MRF4, control the expression of several muscle specific genes (Murre et al. 1989). During embryogenesis, MRFs are responsible for stem cell proliferation and differentiation to form muscle fibers (Kronnie and Reggiani 2002). MyoD and myogenin may also be involved in establishing and maintaining of slow and fast mature muscle fibre phenotype; myogenin is expressed at higher levels compared with MyoD in slow muscles, whereas the opposite is true for fast muscles (Lin et al. 1991; Hughes et al. 1993; Voytik et al. 1993; Allen et al. 2001; Alway et al. 2001).

Although the MRFs are involved in many muscle ontogenetic processes, interestingly, MyoD and myogenin were unaffected in SOL and EDL muscles of young and adult offspring subjected to maternal protein restriction. This result is intriguing because we observed a change in muscle fiber-type frequency in EDL muscle in 30-day-old offspring. However, in this period, myogenin was more expressed than MyoD in both NP and LP groups, with a slight decrease in LP group. We suspect that this fact could

influence the type II muscle fiber differentiation during the maternal protein restriction imposed in our experiment. However, at the end of the experiment, EDL fiber frequency was similar between the groups, and the MyoD expression was high in both groups comparing to myogenin. This fact confirms the implication of the MyoD and myogenin in the differentiation and maintenance of muscle phenotype.

In conclusion, our results indicate that maternal protein restriction followed by normal diet after birth induced morphological changes in muscles with distinct morphofunctional characteristics over the long term, but did not alter the MRFs MyoD and myogenin expression. Further studies are necessary to better understand the mechanisms underlying the maternal protein restriction response on skeletal muscle.

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