

Maternal Undernutrition Alters The Skeletal Muscle Development and Methylation of Myogenic Factors in Progeny: A Randomized Controlled Trial

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Abstract

Maternal malnutrition alters protein synthesis and muscle development in offspring. Epigenetic modification is one of the mechanisms involved in this developmental programming. However, the role of the methylation of myogenic factors remains unclear. Twenty-four pregnant goats were assigned to a control (100% of the nutrients requirement, $n = 12$) or a restricted group (60% of the nutrients requirement, $n = 12$) between 0.3 and 0.7 gestation (G). Descendants were harvested at 0.7 G and at 91 days after birth to analyze the fiber area and fiber type of femoris muscle and to examine the effects on the mTOR signal pathway and methylation of the promoter regions of myogenic factors. Maternal undernutrition increased the fiber area of the vastus muscle in the fetuses ($P < 0.05$). Undernutrition enhanced ($P < 0.01$) the proportions of MyHCI and MyHCIIA fibers in the vastus muscle of fetuses and semitendinosus (ST) muscle of kids, while the proportion of MyHCIIIX fibers were decreased ($P < 0.01$) in the restricted fetuses and kids. The mRNA expression levels of *EYA1* and *MYOZ2* in the restricted fetuses were downregulated ($P < 0.05$), but no difference was observed in the restricted kids ($P > 0.05$). The percentage of DNA methylation at the + 530 CpG site of the *MYF5* gene in the restricted fetuses was increased ($P < 0.05$), but the methylation percentages of the *MYF5* gene at the +274,280 CpG site and of the *MYOD* gene at the +252 CpG site in the restricted kids were reduced ($P < 0.05$). The mTOR protein signals were downregulated ($P < 0.05$) in the restricted fetuses and kids. These results indicated that maternal undernutrition altered the fiber type of femoris muscle in offspring, but its relationship with the methylation in the promoter regions of myogenic genes needs to be further studied.

Background

During myogenesis, the numbers of myoblast, primary muscle fiber and secondary muscle fiber are determined before birth in humans [1] and domestic animals [2, 3]. The skeletal muscle is highly susceptible to nutritional deficiencies due to the low priority of nutrient acquisition in mammals [4, 5]. Nutritional deficiency in early gestation lessens the number of primary muscle fibers [6], but the effects are generally minor due to low nutrients requirement by early fetuses. Secondary muscle fiber proliferates from around 0.3 G (30% of gestation stage), and this process ceases at about 0.7 G [1-3], which constitutes nearly 95% of the final fiber population [2]. Insufficient nutrition during midgestation (0.3 - 0.7 G) affects the number, size, and type of secondary fibers before [2, 7] and after birth [8-10]. It is widely accepted that skeletal muscle development is nutritionally programmed *in utero* [4, 5], and understanding the underlying mechanisms of this programming helps to prevent nutritional sarcopenia in humans and improve meat production in livestock.

Alteration of muscle phenotype can partly be interpreted by the changes in hormone response [11], mTOR signaling pathway [7], myogenic regulatory factors expression [12, 13], and mitochondrial function [14] in model animals. However, an unresolved question is how this phenotype is programmed during ontogenetic muscle development. Epigenetic modification is one of the key mechanisms responsible for the persistent programming [15]. Myogenic factors including MYOD, myogenin, MYF5, and MYF6 (or MRF4) govern skeletal muscle determination and differentiation [16], among which MYF5 and MYOD are the master regulators. Epigenetic modification, including DNA methylation of enhancer chromatin [17, 18], histone acetylation, and long non-coding RNA in mice [19, 20] of the *MYF5* and *MYOD* genes, is associated with the expression of muscle-specific genes during myogenesis and differentiation. Methylation of the promoter region is a regulation target in intrauterine skeletal programming, for example, the *GLUT4* promoter in pigs [21]. However, the relationship

between methylation of the promoter regions of the *Myf5* and *MYOD* genes and the muscle developmental programming under nutrient stress remains uncharacterized. DNA methylation generally reduces the expression of muscle-related genes [22]. We thus hypothesized that maternal undernutrition during midgestation would decrease the expression of *MYF5* and *MYOD* genes via increasing the methylation of their promoter regions.

Small ruminants are optimum animal models for intrauterine programming [23], and 40% energy restriction in pregnant goats leads to hepatic metabolic programming in offspring in our previous study [24]. In the present study, we investigated the effects of 40% maternal undernutrition during mid-gestation on the muscle fiber histology, myosin heavy chain expression, methylation modification of myogenic factors, and the mTOR signal pathway in the skeletal muscles of prenatal and postnatal goats.

Methods

Ethical approval

All of the protocols for this study were approved by the Animal Care Committee according to the Animal Care and the Use Guidelines of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China (No. KYNEAAM-2015-0009).

Experimental design and animal management

Twenty-four goats (45 ± 3 d of gestation, Liuyang black goats, a local breed) were selected and tested by portable ultrasonography (Aloka SSD-500 with a 5-MHz linear probe, Aloka, Shanghai, China). Dams were randomly assigned into the control [100% of the nutrients requirements suggested in the Chinese Meat Goat Requirements (2004), $n = 12$] or restricted group (60% of the nutrients requirements, $n = 12$) according to body weight (BW) and litter size. All dams were placed within individual pens and fed a diet twice (0800 h and 1600 h) per day with a 50:50 ratio of concentrate to roughage, with free access to drinking water. The ingredients and composition of the experimental diet on a dry matter basis are shown in additional Table S1. The feeding of the restricted group was conducted by providing 60% of the feed allowance of the control group during days 45 - 100 of gestation, and the actual restriction level (1.04 kg/d for the control group vs 0.62 kg/d for the restricted group) was 60.2% after measurement of the daily feed allowance and refusal. At day 100 of gestation, six pregnant dams per group were selected for harvesting, and ten fetuses (three singletons, two sets of twins, and one set of triplets) in each group were obtained. The ratio of females to males was 7:3 in the control group and 6:4 in the restricted group.

After day 100 of gestation, feed restriction was removed, and the remaining dams were fed to supply 100% of the nutrients requirements and were managed as before during the following experimental period. After parturition, neonatal kids were nursed by their dams until preweaning between days 50 and 60. Preweaning was conducted by separating offspring from their dams in the daytime from 0800 to 1600 h, and a mixed diet of starter and fresh *Miscanthus* spp. was provided at a ratio of 20:80 during this period. Complete weaning was performed on day 60. Then, all kids were given *ad libitum* access to the above diet and had free access to drinking water. The ingredients and composition of the kid diet are shown in a Table S1. At 90 days of age, eight kids in each group were obtained for harvesting. The ratio of females to males was 3:5 in the control and 4:4 in the restricted group.

Body weight measurement and muscle tissue sampling

At day 100 of gestation and at day 90 after birth, the feeding of all selected animals was withdrawn for 24 h, while clear water was offered freely. The empty BW of fetuses and kids were reported previously [24]. Following electric shock and exsanguination, samples of semitendinosus (ST) and vastus lateralis (VL) muscle were collected from the kids, while the gross vastus muscle in the fetuses was sampled because it was difficult to dissect into individual muscles. The sliced tissue samples were immersed in 10% formalin fixing solution for histology measurement, while another aliquot of samples was quick-frozen in liquid nitrogen and then stored at -80°C for further analysis.

Histology

After fixation, the muscle samples were embedded in paraffin, sliced at a thickness of 8 µm using a rotary microtome (RM2016, Leica Microsystems Inc., Wetzlar, Germany), and stained with hematoxylin (AS1055A, Aspen Biological, Inc., Wuhan, China) and eosin (AS1094, Aspen Biological, Inc.) according to Zou et al. [12]. Ten different microscopic fields of each section and five sections per sample were randomly chosen to determine the muscle fiber area using the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, USA).

Quantitative real-time PCR

The extraction and qualification of total RNA and the analyses of messenger RNA (mRNA) expression levels were performed using the SYBR green-based method with gene-specific primers (Table 1) according to Zhou et al. [24]. A melt curve analysis was conducted to confirm specific product amplification. *ACTG1* was used as the reference gene, and the real-time PCR data were calculated using the $2^{-\Delta\Delta Ct}$ method [25]. The expression of mRNA was presented as the fold change relative to the reference gene.

Immunoblotting analysis

The extraction and qualification of total protein and the immunoblotting analysis were carried out according to the method described [26]. The primary antibodies of MyHCI (MYH7, No. MFCD00162703, Merck KGaA, Darmstadt, Germany), MyHC-IIa (MYH2, No. ab124937, Abcam plc. Cambridge, USA), MyHC-IIx (No. BM0096, Boster Biological Technology Co. Ltd, California, USA), and β -tubulin (No. 2146S, Cell Signaling Technology, Inc., Danvers, UK) were diluted according to 1:5000, 1:10000, 1:100 and 1:1000, respectively. The primary antibodies of AKT (No. AV06008-100UL, Merck KGaA, Darmstadt, Germany), p-AKT (No. 9275, Cell Signaling Technology, Inc., Danvers, MA, USA), PI3K (No. C312573, LifeSpan BioSciences, Inc., Seattle, WA, USA), p-PI3K (No. C358831, LifeSpan BioSciences, Inc.), mTOR (No. PLA0114, Merck KGaA), p-mTOR (No. 2971, Cell Signaling Technology, Inc.), and β -Actin (No. 4976, Cell Signaling Technology, Inc.) were diluted as 1/1000. The density of bands was quantified and then normalized to the reference protein of β -tubulin or β -Actin. The normalized values were used for comparison of the relative expression levels of the target proteins between the control group and the restricted group.

DNA methylation detection

Approximately 20 mg of frozen sample was ground in liquid nitrogen, and DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantitative

methylation analysis of multiple CpG sites was performed by the Beijing Genomics Institute (Guangzhou, China) using a Sequenom EpiTYPE system based on MALDI-TOF mass spectrometry according to Suchiman et al. [27]. The CpG-rich sequences for *MYF5* and *MYOD* were selected using EMBOSS Cpgplot (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/), and one CpG island was found in the primer regions of *MYF5* and *MYOD*. The CpG island of *MYF5* ranges from the site of -10 to +347 bp relative to the TSS site (10070240 - 10070597 in chromosome 5). The forward primer sequence of *MYF5* was TTTATTTTGGGTAGTTTTTGGTTAGG tagged with the T7-promoter of aggaagagag, while the reverse sequence was CCCAAAAATATATAAAAAACCCCAA tagged with the sequence of cagtaatagcactcactatagggagaaggct. The product size was 558 bp from -44 to + 503 (10070196 to 10070753 in chromosome 5) and covered 41 CpG sites, among which 34 CpGs could be effectively quantified. The CpG island of *MYOD* ranges from the site of +159 to + 888 relative to the TSS site (47532862 to 47533591 in chromosome 15). The forward primer sequence of *MYOD* was TAGTTTTGGGAGTTTAGTGTGAAGG tagged with the T7-promoter of aggaagagag, while the reverse sequence was CCTTACAAACCCACAATAAACA tagged with cagtaatagcactcactatagggagaaggct. The product size was 546 bp from -15 to + 530 (47532688 - 47533233 in chromosome 15) and covered 55 CpG sites, among which 42 CpGs could be effectively quantified. The spectral data were pre-processed and analyzed according to the method of Suchiman et al. [27]. The methylation level of the restricted group in each CpG site was expressed as the value relative to the control group.

Statistical analysis

Data were analyzed by a mixed model with treatment, gender and litter size as fixed factors, and initial BW of dams as the covariate. Statistical significance was considered at $P < 0.05$, and Sidak method was applied to compare means. All results are presented as the means and standard errors (SE).

Results

Body weight (BW) and area of muscle fibers

The effect of maternal undernutrition on the muscle fiber area of fetuses and kids is presented in Fig. 1. The BW of fetuses were unaffected ($P > 0.05$) by undernutrition, but the BW of restricted kids was decreased relative to controls ($P = 0.027$). The muscle fiber area of the vastus muscle of restricted fetuses was increased ($P = 0.032$), while it was unaffected ($P > 0.05$) in the ST and VL muscles of restricted kids. Gender and litter size did not affect the area of muscle fibers in the fetuses or kids ($P > 0.05$).

Myosin heavy chain expression

The effect of maternal undernutrition on the expression of myosin heavy chain in the muscle tissues of fetuses and kids is presented in Fig. 2. The expression of *MyHCII*B mRNA in the fetuses and kids was not detectable, so the expression of MyHCII B protein in these samples was not analyzed further. Compared to the control group, maternal undernutrition did not change ($P > 0.05$) the expression of *MyHCI*, *MyHCII*A, or *MyHCII*X mRNA in the vastus muscles of fetuses (Fig. 2A), or in the ST (Fig. 2B) and VL (Fig. 2C) muscles of kids. However, the protein expression levels of MyHCI and MyHCII A were increased ($P < 0.05$) both in the vastus muscles of fetuses (Fig. 2D) and in the ST muscles of kids (Fig. 2E), while the MyHCII X protein expression in the fetuses and in the ST muscles of kids was decreased ($P < 0.01$). Protein expression levels of MyHCI, MyHCII A, and MyHCII X in the VL

muscles of kids (Fig. 2F) were not affected ($P > 0.05$). Gender and litter size did not affect ($P > 0.05$) the mRNA or protein expression of MyHCI, MyHCIIA, and MyHCIIIX genes in the fetuses or kids.

mRNA expression and DNA methylation of myogenic factors

Since no significant differences in myosin heavy chain expression of muscle resulting from maternal undernutrition were observed, we further determined the mRNA expression and DNA methylation of myogenic factors in the muscles of fetuses and kids (Fig. 3). Maternal undernutrition did not influence ($P > 0.05$) the expression of *MYF5*, *MYF6*, *MYOD*, or *MYOG* mRNA in the vastus muscles of fetuses (Fig. 3A) or in the ST muscles of kids (Fig. 3B). The expression levels of *EYA1* ($P = 0.044$) and *MYOZ2* ($P = 0.038$) mRNA in the vastus muscles of restricted fetuses were downregulated, while no difference was observed in the ST muscles of kids ($P > 0.05$). Maternal undernutrition increased ($P = 0.040$) the methylation level of +530 CpG site of *MYOD* gene in the fetuses, while the methylation levels of the +274, 280 CpG site of the *MYF5* gene and the +252 CpG site of the *MYOD* gene in the restricted kids were decreased ($P < 0.05$). The methylation levels of the remaining CpG sites of *MYOD* and *MYF5* genes were not affected ($P > 0.05$) in the fetuses or kids. Gender and litter size did not affect ($P > 0.05$) the mRNA expression of these genes or the CpG methylation of *MYF5* and *MYOD* promoters in the fetuses. Gender affected ($P < 0.05$) the *MYF5* and *SIX1* mRNA expression in kids, which were greater in the females than that in the males. And the methylation of the *MYF5* +32 CpG site in the female kids was higher ($P = 0.041$) than that of the males.

mTOR Signaling Pathway

Maternal undernutrition reduced ($P < 0.01$) the protein expression of mTOR, p-mTOR, and the ratio of p-mTOR/mTOR in fetal vastus muscle. Maternal undernutrition also reduced ($P = 0.037$) the mTOR protein expression in kids and tended to reduce ($P = 0.088$) the PI3K phosphorylation level, while the effects on the PI3K, AKT, and p-AKT proteins and the ratio of p-AKT/AKT in kids were not significant ($P > 0.05$). Gender and litter size did not affect the above protein expression in the fetuses or kids ($P > 0.05$).

Discussion

During the past two decades, there has been increased research interest in the effect of maternal undernutrition on muscle development of offspring. In this study, maternal undernutrition altered the muscle fibers and composition of the vastus muscle in fetuses and kids, and downregulated the mRNA expression of myogenic factors in the fetuses. DNA methylation levels of several sites in the *MYF5* and *MYOD* promoter regions in the fetuses and kids were also affected by maternal undernutrition. Many studies have confirmed that maternal undernutrition in the second trimester is closely associated with the thrifty phenotype of offspring in human epidemiological surveys [28, 29] and in animal model trials [8, 30, 31]. In the present study, no changes in the BW of fetuses and the diminished BW in the restricted kids were consistent with a compensation effect by the mother *in utero* and a thrifty phenotype after birth. However, the increment of muscle fiber area in restricted fetuses in this study was not consistent with the findings (no significant change) in the fetuses of cattle [13] and pigs [12] during midgestation or in sheep (the decreased size) [7]. According to Wigmore and Stickland [2], the primary fibers begin to lose the central myofibril-free region at around 0.65 G, and then the diameter of the muscle fibers decreases rapidly. The nutrient-restricted fetuses can prolong the maturation of the primary muscle fibers and delay the time of disappearance of the central myofibril-free cytoplasm. In this study, the

observation period of fetal muscle fibers coincided with this stage (0.67G), and this may be the reason for the increase in the area of muscle fibers of the restricted fetuses.

Skeletal muscle is generally divided into four types according to myosin heavy chains (MyHC): slow-twitched type I (MyHCI), fast-twitched type IIA (MyHCIIA), IIB (MyHCIIIB), and IIX (MyHCIIIX). Previously, Zhu et al. [10] reported that 50% maternal undernutrition increased the MyHCIIIB ratio and decreased the percentage of MyHCIIA of the longissimus dorsi (LD) muscle in the restricted lambs at eight months of age, while the type composition of LD and ST muscles in the 50% restricted lambs at 150 days of age was unaffected [8]. In contrast, Fahey et al. [32] reported fewer fast fibers and more slow fibers in the LD and VL muscles of 50% maternally restricted 14-day-old lambs. In this study, the types of MyHCI and MyHCIIA were increased, but MyHCIIIX was decreased both in the vastus muscle of 40% restricted fetuses and in the ST muscles of restricted kids, while the VL muscles in offspring were not affected. These results suggest that the effects of similar maternal undernutrition during midgestation on the muscle fiber development vary, and the cause needs further research. Moreover, comparing LD and ST fibers from the same muscle position, a similar degree of feeding restriction (such as 50%) exerted different effects on muscle fiber development [8, 10, 32]. It has been speculated that this discrepancy is related not only to the muscle fiber composition in different muscle positions but also to the timing of phenotypic plasticity induced by different types of nutrient restriction [33, 34].

In this study, we noted an inconsistency in the expression between mRNA and protein levels of MyHC isoforms in the fetal vastus and kid ST muscles. Similar differences in mRNA and protein abundances of MyHC genes have been reported in other species [35]. Generally, the protein accretion rate of hindlimb muscle is approximately 0.17% per day in ovine fetuses [36] and 0.8% in young lambs [37]. However, transcriptional and posttranscriptional regulation of *MyHC* isoforms mRNA in muscle tissue is active and sensitive to environmental cues (e.g., hormones or metabolites) [38, 39]. MyHC expression at the protein level represents the existing fiber type composition within the muscle. Therefore, the effect of protein expression was highlighted.

Consistent changes in the MyHC types in the vastus muscle and the ST muscle of the goats hinted that metabolic programming could have taken place in the present study. DNA methylation is one of the universal mechanisms regulating this phenotypic change [40]. *MYF5* and *MYOD* gene methylation is important in regulating muscle differentiation. Increased methylation of three important CpG sites in the *MYOD1* gene and decreased *MYOD1* mRNA expression have been observed during the differentiation of human primary muscle fibers from myoblasts to myotubes, while the methylation and mRNA levels of the *MYF5* gene were decreased [41]. However, there are few reports on the methylation modification of these two MRF factors under intrauterine nutrition restriction. Our results revealed that maternal nutritional restriction during midgestation affected the methylation of individual sites in the *MYOD* and *MYF5* promoter regions of the fetuses and kids. These affected sites may not be closely correlated with the regulation of *MYOD* and *MYF5* mRNA expression, because the mRNA expression of *MYOD* and *MYF5* was unaffected. Previous studies have shown that maternal low-amino acid diets in the middle and late stages of pregnancy reduced the methylation level of the CpG island in *IGF2R* gene intron 2 in the longissimus muscles of sheep fetuses at 130 days [42]. A similar study found that the average CpG island methylation in the *PGC1A* promoter sequence in the skeletal muscle of intruterine growth retardation rats with protein restriction during pregnancy increased, while the GLUT4 and *PGC1A* mRNA expression and glucose tolerance decreased [43]. In our study, we have not yet identified the key methylation modification sites that can explain the phenotypic changes in offspring, thus further research is needed.

The mRNA expression levels of myogenic factors *MYOG* and *MRF4* in fetuses and kids were unaffected, but the levels of *EYA1* and *MYOZ1* mRNA were decreased in the restricted fetuses. *SIX* and *EYA1* genes are specifically involved in the differentiation of limb buds [44]. The *SIX1* and *EYA1* proteins accumulate preferentially in the nuclei of fast-twitch muscles and induce a fiber-type transition with the replacement of myosin heavy chain I and IIA isoforms by the faster IIB and/or IIX isoforms [45]. Furthermore, *MYOZ2* is expressed in slow-twitch skeletal muscle, and the downregulation of *MYOZ2* is associated with increased slow-twitch muscle fibers [46]. The downregulation of *EYA1* and *MYOZ2* mRNA in the restricted fetuses is consistent with the increases in MyHCI and MyHCIIA types and the decrease in MyHCIIx in the present study.

The development of skeletal muscle is a highly complicated process involving nutritional factors and numerous signaling pathways to regulate muscle-specific transcription factors, and mTOR signaling is a key regulator of skeletal muscle development at distinct stages of myogenesis [47]. In this study, mTOR protein was downregulated in the femoris muscles of fetuses and kids. We considered that compared to the normal energy requirement, a 40% maternal undernutrition aggravated the overall lack of proteins and amino acids, resulting in the downregulation of the mTOR signals in the offspring, thereby affecting the protein synthesis of muscle tissues. Furthermore, the mTOR protein controls the muscle-specific miR-1 transcription in mice that regulates the stability of *MYOD* and *MYF5* expression [48]. In addition, other miRNAs such as miR-133, miR-206, and miR125b are also linked to the mTOR pathway to regulate muscle fiber differentiation [48, 49], and the muscle-specific expression of miRNAs can be regulated by nutrients through epigenetic mechanisms [47]. This provides new ideas for studying the mechanism of muscle fiber development programming under maternal nutrition restriction.

Conclusion

The maternal undernutrition during midgestation increased the expression of MyHCI and MyHCIIA proteins in the vastus muscles of fetuses and the ST muscles of kids and reduced MyHCIIx protein expression, hinting at the developmental programming of skeletal muscle fibers. Transcripts of *EYA1* and *MYOZ2* genes in the restricted fetuses were downregulated, and the mTOR protein signals declined in the restricted fetuses and kids. Maternal feeding restriction may prompt the switch from MyHCIIx to MyHCI and IIA in the femoris muscles of fetuses and kids associated with repression of the mTOR pathway. This programming was associated with the reduction of *EYA1* and *MYOZ2* transcripts, but the association between the mRNA expression and the DNA methylation in the promoter region of the myogenic factors *MYF5* and *MYOD* needs to be further investigated.

Abbreviations

MyHCI = myosin heavy chain 1; MyHCIIA = myosin heavy chain IIA; MyHCIIx = myosin heavy chain IIX; MYF5 = myogenic factor 5; MYF6 = myogenic factor 6; MYOD = myogenic differentiation 1; MYOG = myogenin, *EYA1* = eyes absent homolog 1; *SIX 1* = homeobox protein *SIX1*; *MYOZ1* = myozenin 1; *MYOZ2* = myozenin 2; GLUT4 = glucose transporter type 4.

Declarations

Ethics approval and consent to participate

All of the protocols for this study were approved by the Animal Care Committee according to the Animal Care and the Use Guidelines of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China (No. KYNEAAM-2015-0009).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XZ: Investigation, Data collection, Writing; **QY:** Project administration, Conceptualization, Investigation; **LL:** Data Curation; **GC:** Data collection; **ST:** Data analysis; **ZH:** Visualization; **ZT:** Conceptualization, Funding acquisition. All authors read and approved the final manuscript.

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Tables

Table 1 Primer sequences used for quantitative RT-PCR

| Gene | Forward (5' to 3') | Reverse (5' to 3') | Product length (bp) | Locus |
|----------------|----------------------|-----------------------|---------------------|--------------------------------|
| <i>MyHCI</i> | ACCAACCTGTCCAAGTTCCG | CGCGGCTACTCCTCATTCAA | 143 | XM_018054604.1 |
| <i>MyHCIIA</i> | AAGGGCTGACATTGCTGAGT | TGCCTCTCTTCAGTCATTCCA | 122 | XM_018064659.1 |
| <i>MyHCIIX</i> | GGTCTACGCAAACACGAGAG | GCGGAATTTGGAGAGGTTGAC | 177 | XM_018064657.1 |
| <i>MYF5</i> | AGACGCCTGAAGAAGGTCAA | CTCCACCTGTTCCCTTAGCA | 150 | NM_001287037.1 |
| <i>MYF6</i> | CAAGTCAGAGGCCAAGGAAG | TTCTAAGGGCTGCAGGGTAA | 103 | NM_001285602.1 |
| <i>MYOD</i> | TGCAAACGCAAGACGACTAA | CTGGTTTGGGTTGCTAGACG | 126 | XM_018058990.1 |
| <i>MYOG</i> | ACAATCTGCACTCCCTCACC | CATCCTGGCAGACAATCTCA | 106 | NM_001285733.1 |
| <i>EYA1</i> | CCACTCATGTCCAGCTCAGA | GACTGCGAGGCTGTAAACC | 137 | XM_013967225.2 |
| <i>SIX1</i> | CAGTCACCTCGCACTTTGAA | TCCTTCATTTCCACAGAGG | 160 | XM_018058424.1 |
| <i>MYOZ1</i> | GGACAGCAATGCCTTATGGT | AACTAAGGGTTCGCTCAGCA | 101 | XM_005699215.3 |
| <i>MYOZ2</i> | TGCCATGCAGAATGAGAAAC | TAGGGACAGCTGTGGTGTG | 182 | XM_013964451.2 |
| <i>ACTG1</i> | ATGGCTACTGCTGCGTCGT | TTGAAGGTGGTCTCGTGGAT | 161 | XM_018063603.1 |

Figures

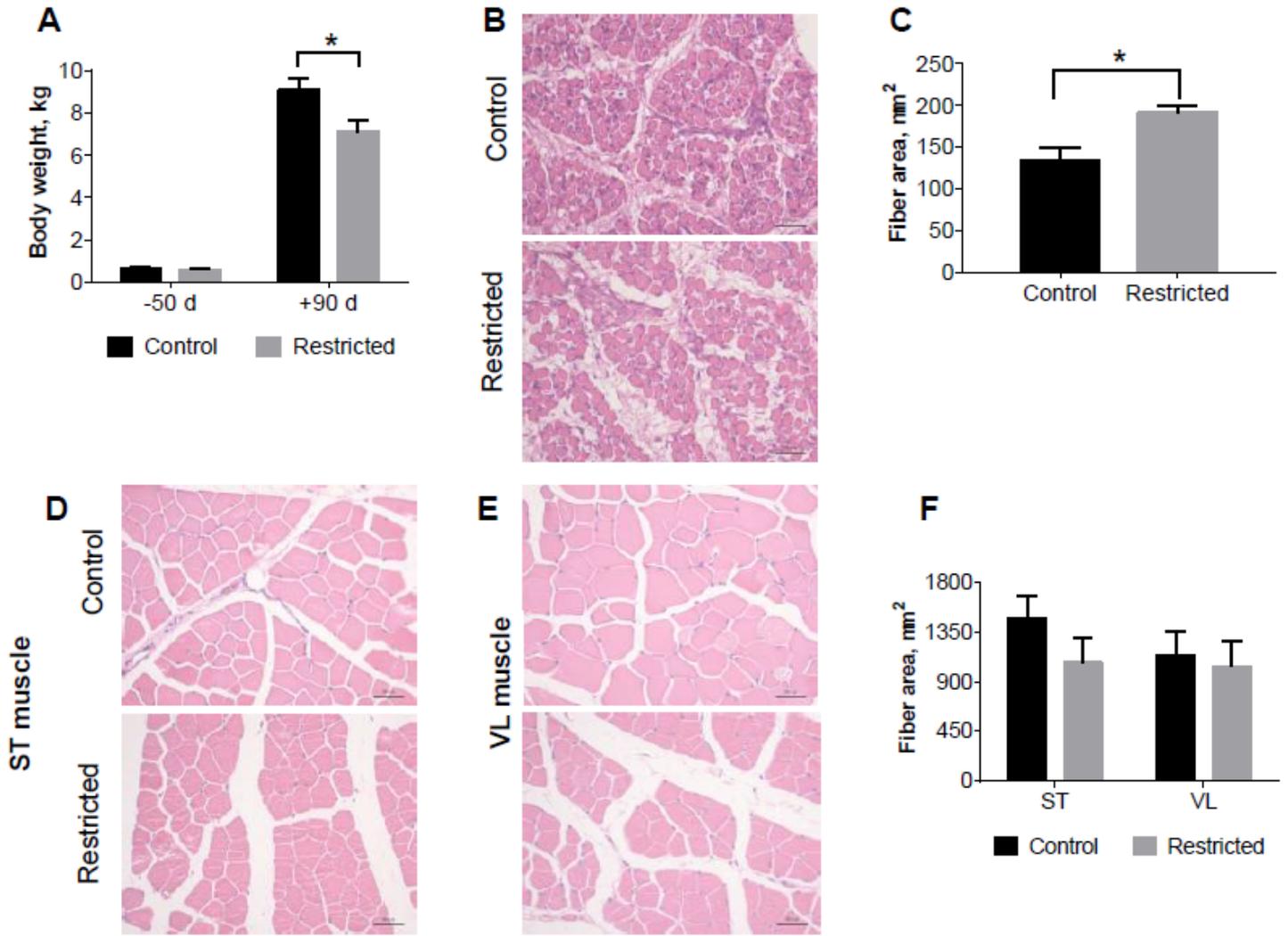


Figure 1

Body weight and area of muscle fibers in offspring. (A) Body weight; Histological section (B) and fiber area (C) of the vastus muscles in fetuses; Histological section of semitendinosus (ST, D) muscles and vastus lateralis (VL, E) muscles in kids; (F) Fiber areas of ST and VL muscles in kids. *P < 0.05.

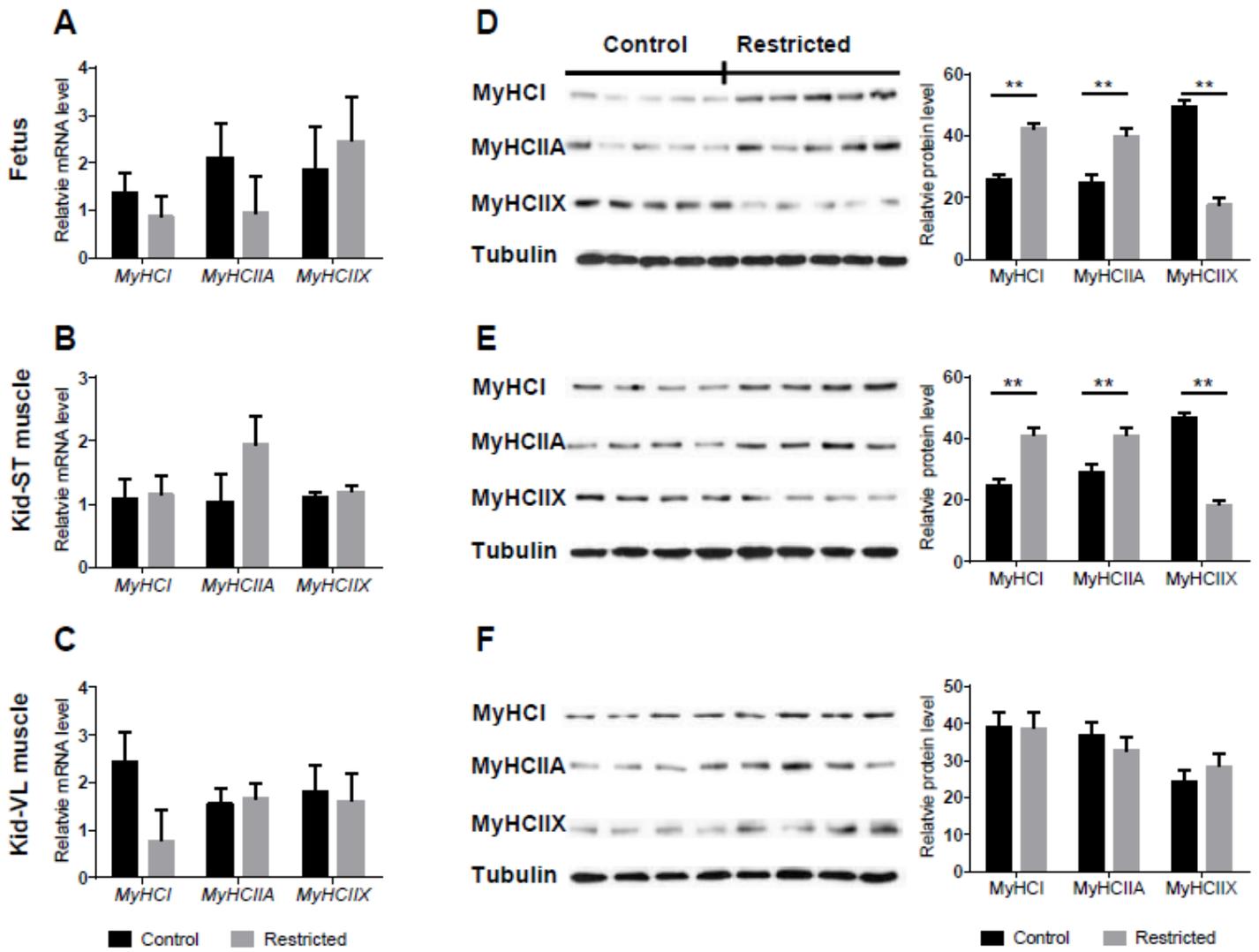


Figure 2

mRNA and protein expression of myosin heavy chain in offspring. (A) mRNA expression in the vastus muscle of fetuses; mRNA expression in the semitendinosus (ST, B) and vastus lateralis (VL, C) muscles of kids; (D) Protein expression in the vastus muscle of fetuses; Protein expression in the ST (E) and VL (F) muscles in kids. *P < 0.05; **P < 0.01.

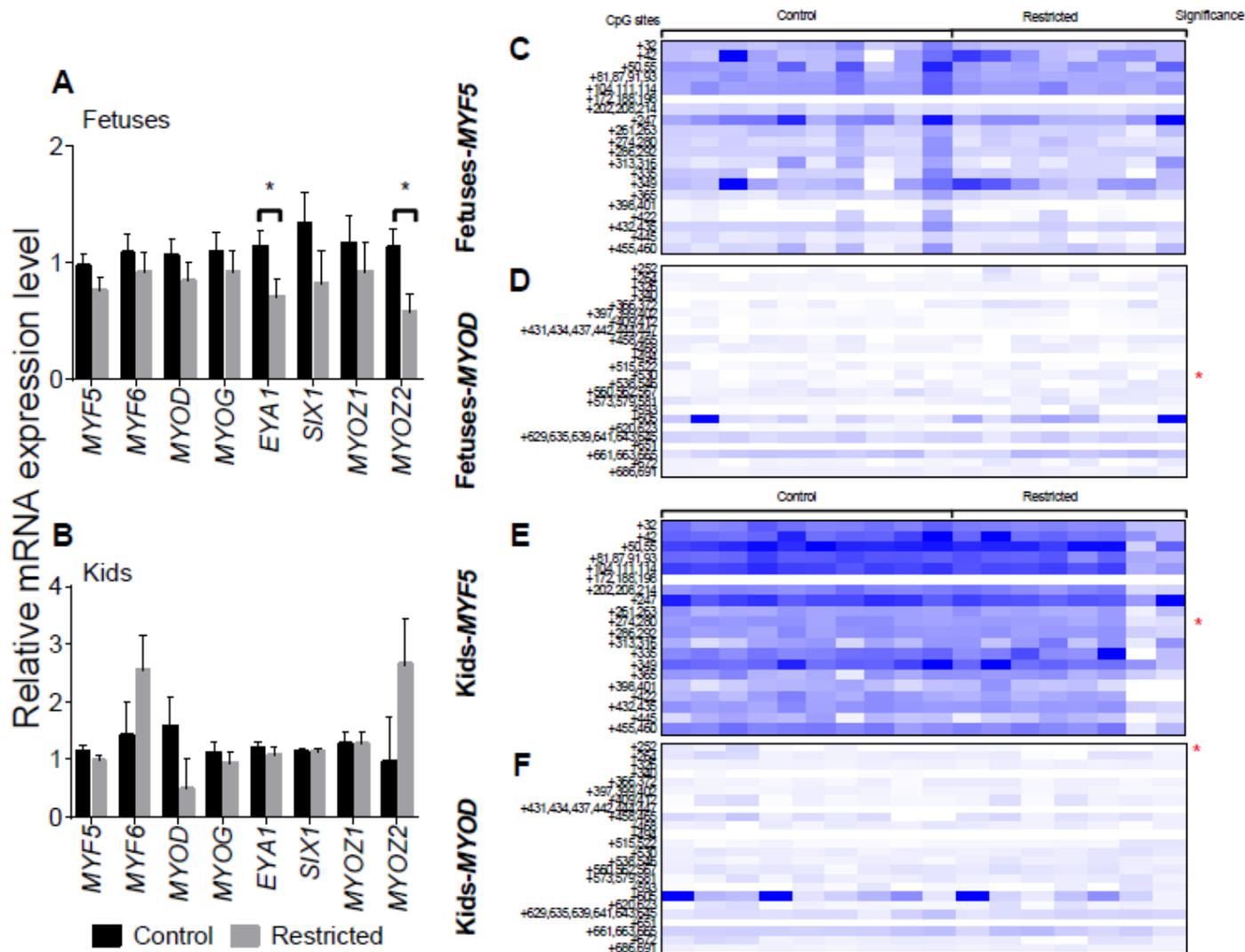


Figure 3

The mRNA expression and DNA methylation of myogenic factors in offspring. (A) mRNA expression in the vastus muscles of fetuses; (B) mRNA expression in the semitendinosus (ST) muscles of kids; (C) MYF5 and (D) MYOD methylation in the vastus muscles of fetuses; (E) MYF5 and (F) MYOD methylation in the semitendinosus (ST) muscles of kids. *P < 0.05.

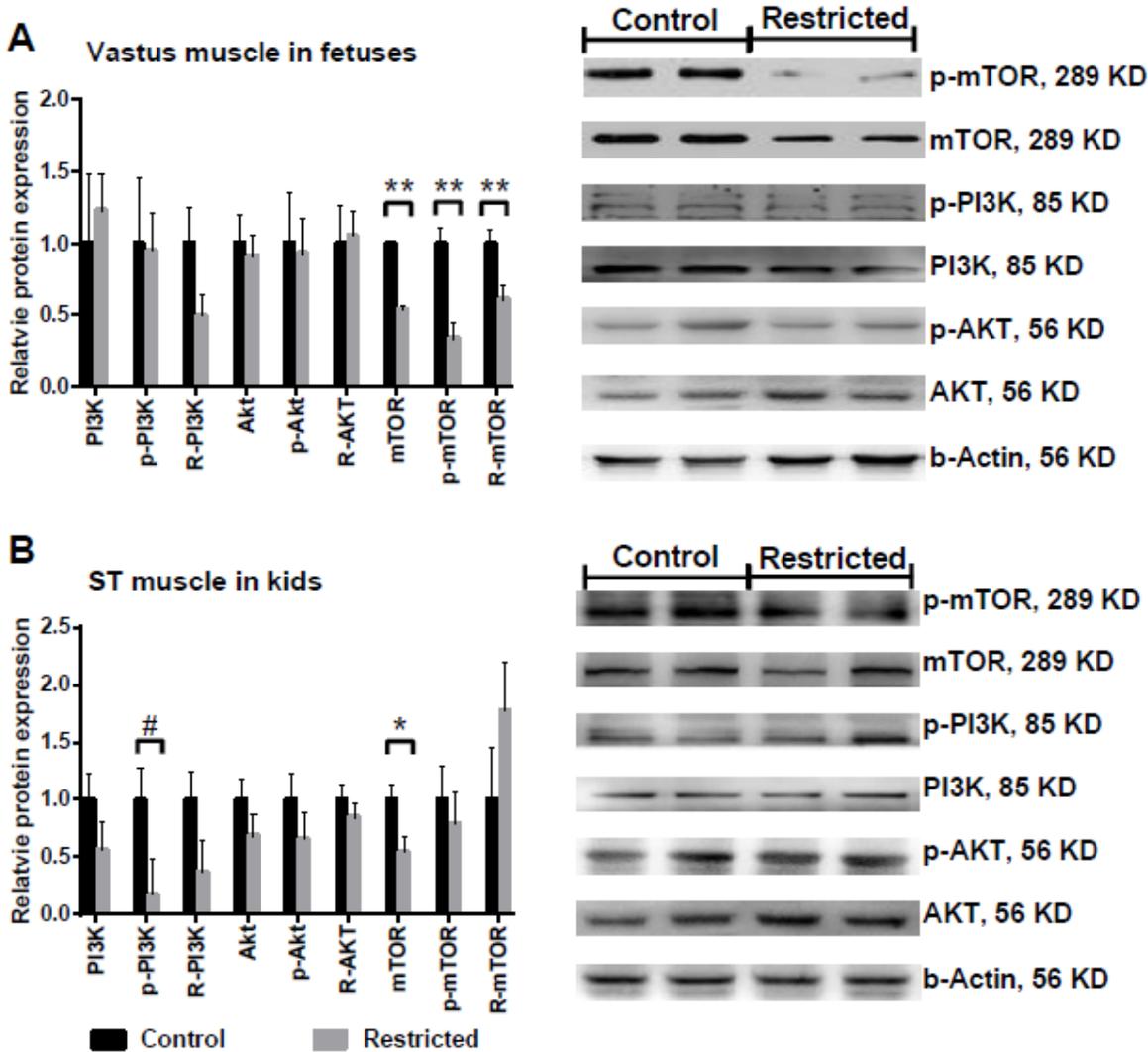


Figure 4

The protein expression of mTOR signaling in the skeletal muscles of offspring. (A) vastus muscles in fetuses; (B) semitendinosus (ST) muscles in kids. *P < 0.05; **P < 0.01.

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