



Contents lists available at ScienceDirect

## Animal Nutrition

journal homepage: <http://www.keaipublishing.com/en/journals/aninu/>

## Original Research Article

# Maternal intake restriction programs the energy metabolism, clock circadian regulator and mTOR signals in the skeletal muscles of goat offspring probably via the protein kinase A-cAMP-responsive element-binding proteins pathway



Xiaoling Zhou<sup>a, b, c, 1</sup>, Qiongxian Yan<sup>a, d, 1</sup>, Hong Yang<sup>a</sup>, Ao Ren<sup>a</sup>, Zhixiong He<sup>a</sup>, Zhiliang Tan<sup>a, \*</sup>

<sup>a</sup> CAS Key Laboratory for Agro-Ecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Hunan Provincial Key Laboratory of Animal Nutrition & Physiology and Metabolism, South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, 410125, China

<sup>b</sup> University of the Chinese Academy of Science, Beijing, 100049, China

<sup>c</sup> College of Animal Science, Tarim University, Alaer, 843300, China

<sup>d</sup> Hunan Co-Innovation Center for Utilization of Botanical Functional Ingredients, Changsha, 410128, China

## ARTICLE INFO

## Article history:

Received 29 December 2020

Received in revised form

25 August 2021

Accepted 8 September 2021

Available online 6 October 2021

## Keywords:

Maternal effect

Skeletal muscle

Fat oxidation

Protein synthesis

Circadian clock

Goat

## ABSTRACT

The biological mechanism by which maternal undernutrition increases the metabolic disorder risk of skeletal muscles in offspring is not fully understood. We hypothesize that maternal intake restriction influences metabolic signals in the skeletal muscles of offspring via a glucagon-mediated pathway. Twenty-four pregnant goats were assigned to the control group (100% of the nutrients requirement,  $n = 12$ ) and restricted group (60% of the control feed allowance from pregnant days 45 to 100,  $n = 12$ ). Blood and *Longissimus thoracis* muscle were sampled from dams (100 d of gestation), fetuses (100 d of gestation), and kids (90 d after birth) in each group. The data were analyzed using the linear MIXED model, with the multiple comparison method of SIDAK applied. Intake restriction reduced ( $P < 0.05$ ) the total blood protein of dams and fetuses. Maternal restriction decreased ( $P < 0.05$ ) the cAMP-responsive element-binding protein 1 (*CREB1*), CREB-binding protein (*CREBBP*), protein kinase A (*PKA*), aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*), protein kinase B (*AKT1*), mammalian target of rapamycin (*mTOR*), and regulatory-associated protein of mTOR (*RPTOR*) mRNA expression in the fetuses, and reduced ( $P < 0.05$ ) the *CREBBP*, nuclear receptor subfamily 1 group H member 3 (*NR1H3*), D-box binding PAR bZIP transcription factor (*DBP*) and *PKA* mRNA levels in the kids, but increased ( $P < 0.05$ ) the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC1A*) and tuberous sclerosis 2 (*TSC2*) mRNA levels in the fetuses. The mRNA expression of clock circadian regulator (*CLOCK*) and *TSC2* genes was increased ( $P < 0.05$ ) in the restricted kids. The protein expression of total *PKA* and phosphorylated *PKA* in the restricted fetuses and kids were downregulated ( $P < 0.05$ ), and the protein expression of total *mTOR* and phosphorylated *mTOR* were reduced ( $P < 0.05$ ) in the restricted fetuses and kids. Maternal intake restriction regulated fat oxidation, protein synthesis, and circadian clock expression in the muscles of the offspring probably via the glucagon-mediated *PKA*-*CREB* pathway, which reveals a

\* Corresponding author.

E-mail address: [zltan@isa.ac.cn](mailto:zltan@isa.ac.cn) (Z. Tan).<sup>1</sup> These authors contributed equally to this work.

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



<https://doi.org/10.1016/j.aninu.2021.09.006>

2405-6545/© 2021 Chinese Association of Animal Science and Veterinary Medicine. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

noteworthy molecular pathway that maternal undernutrition leads to metabolic adaptation of skeletal muscle in offspring.

© 2021 Chinese Association of Animal Science and Veterinary Medicine. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Maternal protein-energy malnutrition often induces a high metabolic risk in offspring (Batool et al., 2015; Hochberg et al., 2011). Skeletal muscle accounts for 40% of the body's weight and is one of the key tissues for metabolic regulation (Beauchamp and Harper, 2015). Maternal undernutrition alters skeletal muscle development by reducing the cell number (muscle fibers), muscle protein synthesis and degradation rates, and muscle fiber type composition and proportion (Gonzalez et al., 2013; Mohrhauser et al., 2015; Sen et al., 2016; Zhu et al., 2006). These alterations affect the metabolic characteristics of skeletal muscle, including insulin sensitivity, glucose transport, uptake, storage and aerobic utilization, and lipid accumulation (Beauchamp and Harper, 2015; Brown and Hay, 2016). The affected offspring are susceptible to imbalances in energy metabolism, insulin resistance, or lipid metabolism disorders in skeletal muscle (Hales and Barker, 2001; Ozanne, 2014).

Muscular energy metabolism is adjusted by a molecular signaling pathway network. The mammalian target of rapamycin (mTOR) pathway is an evolutionarily conserved regulator of cell growth and proliferation that controls intracellular protein anabolism and inhibits protein degradation (Saxton and Sabatini, 2017). It is also one of the central modulators during skeletal muscle development (Yoon, 2017). The AMP-activated protein kinase (AMPK) pathway is in charge of muscle energy sensing and homeostasis (Cui et al., 2017; Hardie, 2011). Moreover, the AMPK pathway is linked to the mTOR pathway via intracellular nutritional signals. One of the signals is ATP availability, and AMPK is activated by an increased intracellular AMP-to-ATP ratio; activated AMPK phosphorylates and activates tuberous sclerosis 1 (TSC1)/TSC2 dimers to inhibit mTORC1 (Hart et al., 2019), thereby coupling the processes of energy sensing and protein synthesis at the cellular level under energy-deficient conditions. Additionally, the rhythmic timing system, including a central clock in the hypothalamic nucleus and peripheral tissue clocks in the liver, muscle and adipose tissue, responds to central and peripheral timing factors such as endocrine hormones, neural signals, eating patterns, body temperature, oxygen, light–dark cycles and sleep–wake signals (Zhang et al., 2010). At the tissue level, the circadian clock integrates intracellular and extracellular signals (such as nutrient levels) to modulate energy homeostasis and protein synthesis (Eckel-Mahan et al., 2012; Reinke and Asher, 2019), and metabolic rhythm disorders are associated with metabolic abnormalities and diseases (Stenvers et al., 2019).

Blood metabolites, amino acids (AA) and hormone secretion reflect the nutritional status of the whole body and are changed in pregnant goats and offspring exposed to late-gestational undernutrition (He et al., 2015). The independent effects of maternal malnutrition on the mTOR signaling (Lie et al., 2015; Zhu et al., 2004) and AMPK pathways (Boyle et al., 2017; Zhu et al., 2004, 2008) in the skeletal muscles of mammals *in vivo* and *in vitro* and on the central circadian clock in rats (Mendonca et al., 2004; Vilela et al., 2005) have been reported. However, knowledge about the association of maternal undernutrition with energy metabolism, protein synthesis, and disruption of the metabolic rhythms of skeletal muscle in offspring is still unknown.

The Liuyang black goat is a local breed in southern China. It can be in estrus year-round and has the reproductive trait of giving birth to multiple kids. Dams often deliver 2 kids, with a maximum of 4 kids per litter (Chen et al., 2021). Female goats are vulnerable to malnutrition during pregnancy. Previously, we noticed an association of an elevated glucagon level in mother goats caused by maternal intake restriction during midgestation with programmed liver energy metabolism in the offspring (Zhou et al., 2019b) and a reduction in muscle mass in kid goats (Zhou et al., 2019a). Glucagon influences tissue metabolism through the classical cAMP-protein kinase A (PKA) - cAMP-responsive element-binding protein 1 (CREB) pathway in peripheral tissues (Goldstein and Hager, 2018), and CREB is a key regulator in energy metabolism (Kim et al., 2017), protein synthesis and circadian control (Wheaton et al., 2018; Wu et al., 2019). To test the hypothesis that maternal intake restriction programs the PKA-CREB pathway of skeletal muscle in offspring to affect metabolic signals, we investigated the effects of 40% maternal intake restriction during midgestation on the concentrations of blood metabolites, muscular cAMP and glycogen, and on the expression of genes involved in the mTOR, AMPK and CLOCK pathways. The findings will provide new insights into the metabolic adaptation caused by maternal undernutrition in humans.

## 2. Materials and methods

### 2.1. Experimental design and animal management

All the protocols used in this study were approved by the Animal Care Committee according to the Animal Care and Use Guidelines of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China (No. KYNEAAM-2015-0009). Twenty-four goats (45 ± 3 d of gestation, Liuyang black goat, local breed) were selected and randomly assigned to a control [CON, 100% of the nutrients requirement suggested in the feeding standard of meat-producing sheep and goats of China (2004),  $n = 12$ , 32.33 ± 3.91 kg] or a feed intake-restricted (RES, 60% of the control,  $n = 12$ , 30.66 ± 3.89 kg) group. All animals were reared in a pen individually during the restriction period from 45 to 100 d of gestation, and then were restored to 100% of the nutrients requirement and fed together until parturition. At 100 d of gestation, after examination with ultrasonography (Aloka SSD-500 with a 5-MHz linear probe Aloka, Shanghai, China), 6 dams and ten fetuses (3 singletons, 4 twins, and 3 triplets) from each group were chosen for slaughtering, and the samples from dams and their fetuses were collected. The ratios of males to females were 7:3 and 6:4 in the CON and RES fetuses, respectively.

The remaining dams were realimented to meet 100% of nutrients requirement after 101 d of pregnancy. After delivery, the newborn goats were naturally breastfed and penned together with their dams in one compartment to prevent cross breastfeeding until 50 d postpartum. All dams were provided with a ration at a 50:50 ratio of roughage to concentrate twice daily at 08:00 and 16:00. The daily DM intakes of the CON and RES dams were 0.93 and 0.65 kg/d during the restriction period, and 1.06 and 1.21 kg/d at the lactation stage, respectively. Between 50 and 60 d of age, the kids were preweaned through separation from their mother

12 h per day. After complete weaning at 60 d of age, the diet containing milk replacer and fresh grass was given twice daily at 08:00 and 16:00 at a ratio of 80:20 until 90 d of age. The DM intake of the kids averaged 0.3 kg/d between 60 and 90 d. At 90 d of age, 8 kids in each group (4 singletons and 4 twins) were slaughtered, and samples were collected. The ratios of males to females were 3:5 and 4:4 in the CON and RES kids, respectively. The ingredients and composition of the experimental diet for the dams and kids are listed in [Appendix Table 1](#).

## 2.2. Blood and tissue sampling

After fasting overnight, the dams and kids were electrically shocked, and blood was collected from the jugular veins. Plasma was anticoagulated with heparin sodium, separated through centrifugation at  $1,200 \times g$  for 10 min at 4 °C and stored at –80 °C for subsequent analysis. Following exsanguination and ventrotomy of the dams, fetal blood samples were collected from the umbilical cord, and the plasma was separated as described above. The hot carcass weight of animals was determined after removal of the skin, head, hoofs, tail and visceral organs but not the kidneys, and samples of the *Longissimus thoracis* (LT) muscle between the 5th and 8th ribs were sliced and snap-frozen in liquid nitrogen and then stored at –80 °C until further analysis.

## 2.3. Blood biochemical parameters and free AA analysis

After plasma samples were thawed at 4 °C, the total protein (TP) concentration was determined using assay kits (Beijing Leadman Biochemistry Company Limited, Beijing, China) with an automatic biochemical analyzer (Hitachi 7600, Hitachi, Ltd., Tokyo, Japan). The concentrations of nonesterified fatty acids (NEFA) were measured using assay kits (Nanjing Senbeijia Biological Technology Co., Ltd., Nanjing, China). The concentrations of triiodothyronine (T3) and thyroxine (T4) were measured using an enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cusabio Biotech Company Limited, Wuhan, China). The intra- and interassay coefficient variations of T3 and T4 were all below 10%.

The analysis of free AA in blood was determined as described earlier ([Chen et al., 2019](#)). Briefly, 500 µL of plasma sample was taken and mixed with 500 µL of 0.30 mol/L sulfosalicylic acid solution. The mixed solution was placed at 4 °C overnight and centrifuged at  $10,000 \times g$  for 10 min at 4 °C. The supernatant was collected and filtered using a 0.22 µm microporous membrane. The filtrate was measured using an L-8800 automatic amino acid analyzer (L-8800, Hitachi, Tokyo, Japan).

## 2.4. Glycogen and cAMP determination

Glycogen (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) and cAMP (NewEast Biosciences, Malvern, USA) in the *Latissimus dorsi* (LT) muscle were determined according to the manufacturer's instructions. The preparation procedure was performed as previously described ([Zhou et al., 2019b](#)), and the results were expressed as mg/g protein in fresh tissue.

## 2.5. Quantitative RT-PCR

The total RNA of the sample was extracted using precooled reagent (TriQuick, Solarbio, Beijing, China) according to the manufacturer's instructions. The expression of the target mRNA was analyzed using the SYBR green method with a PCR platform (LightCycler 480 system, Roche Applied Science, Basel, Switzerland), and the detailed method was described previously ([Zhou et al., 2019b](#)). The specific gene primers are listed in [Table 1](#). Relative gene expression levels

were normalized to the levels of the reference gene actin gamma 1 (*ACTG1*) using the  $2^{-\Delta\Delta Ct}$  method ([Livak and Schmittgen, 2001](#)), where Ct denoted the threshold cycle.

## 2.6. Western blotting

The primary antibodies were against the following: AMPK $\alpha$  (#5831, CST Inc., Danvers, MA, USA), phospho-AMPK $\alpha$  (#2535, CST Inc., p-AMPK $\alpha$ ), serine–threonine kinase 11 (STK11) (ab199970, Abcam plc., Cambridge, CB, UK), phospho-STK11 (#3482, CST Inc., p-STK11), PKA (#4782, CST, Inc.), phospho-PKA (#9621, CST Inc., p-PKA), TSC2 (#4308, CST Inc.), phospho-TSC2 (#3617, CST Inc., p-TSC2), mTOR (#2792, CST Inc.), and phospho-mTOR (#2972, CST Inc.; p-mTOR). The reference protein was GAPDH (ab37168, Abcam plc.). The procedure was conducted according to a previous report ([Chen et al., 2019](#)). The primary antibodies were diluted with 5% BSA at 1:1,000 and incubated overnight at 4 °C. The density of bands was quantified by using BIO-RAD Gel Doc XR+ (Bio-Rad Laboratories, Hercules, CA, USA) and Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA), and the relative expression levels were normalized to the reference protein expression level.

## 2.7. Statistical analysis

The data were analyzed using the linear MIXED model of SPSS 19.0 (IBM SPSS, Inc., 2010). The data from dams were tested with the nutritional level as a fixed effect and the initial weight of the dams as a covariate. All offspring data were analyzed with nutritional level, litter size and offspring sex as fixed factors. After there were significant differences among fixed factors, the multiple comparison method of SIDAK was applied. A significant difference was set at  $P < 0.05$ , and a significant trend was considered at  $0.05 \leq P \leq 0.10$ . The results are expressed as the means and standard errors of the means (SEM).

## 3. Results

### 3.1. Carcass weight and blood biochemical indices

The hot carcass weights of the RES dams and RES kids were less than those of the CON dams ( $P < 0.05$ , [Table 2](#)), but this index was not affected in the fetuses ( $P = 0.182$ ). The NEFA concentrations of the RES dams increased ( $P = 0.007$ ), whereas the TP concentrations of RES dams and RES fetuses decreased ( $P < 0.05$ ). The glucagon concentrations were increased in the RES dams ( $P = 0.002$ ), which was previously reported ([Zhou et al., 2019b](#)).

The TP concentrations of the fetuses and kids ( $P < 0.05$ ) were affected by sex and litter size, which were higher ( $P = 0.045$ ) in the male fetuses (28.87 g/L) than in the females (27.58 g/L), as well as in kids (68.37 g/L in males vs. 62.89 g/L in females,  $P = 0.009$ ). The TP concentration of fetuses was decreased ( $P = 0.007$ ) in sequence in singletons (32.03 g/L), twins (27.56 g/L) and triplets (25.09 g/L), but its concentration in the kids of twins (59.81 g/L) was lower than those in the singletons (69.81 g/L) and triplets (67.26 g/L). The glucagon of fetuses ( $P = 0.018$ ) was affected by litter size, which was increased in order in singletons (31.53 ng/L), twins (32.80 ng/L) and triplets (39.10 ng/L).

### 3.2. Blood AA profile

The Ser concentration of the RES dams and the Thr concentration of the RES kids increased ( $P < 0.05$ , [Table 3](#)), while other detected AA in the dams and kids were not influenced by diet ( $P > 0.05$ ). The Val, Met and Tyr concentrations of the kids were

**Table 1**  
Gene-specific primers for qRT-PCR.

Gene	Primer sequence (5'-3')	Amplicon size, bp	Accession number
<b>Energy metabolism</b>			
<i>ACACA</i>	F: ATGTGGATGATGGGCTGAA R: GCTTGAACCTGTCGGAAGAG	139	XM_018064168.1
<i>ACOX1</i>	F: ACCTGTGAGTTTGTGCTGA R: TTGGGCTGAAAGATGCTAC	109	XM_018063769.1
<i>CPT1A</i>	F: TCATACTCGCTGGGAACAGA R: TCTCGGAAGGAAACAAATGC	111	XM_018043311.1
<i>CREB1</i>	F: TTGTGGTTTTCTAGGTGTGT R: GTTTCTGCTGCTGTGCAAC	115	XM_005676432.3
<i>CREBBP</i>	F: GAATGGATCTCTCGGGCTG R: GCGGTGAAACTTGGGTCAAC	119	XM_018040244.1
<i>G6PDH</i>	F: ACCTATGGCAACCGATACAAGA R: GTGGAGCAGTGGAGTGAAGAT	144	XM_018044343.1
<i>INSR</i>	F: TCAAGGACGGAGTCTTACC R: TTTCAAGCACCTGCTCATTTG	119	XM_018051134.1
<i>NR1H3</i>	F: TGCTGATGAACTGGTGGAGC R: TGAAGACACGGAGGAGGAAC	147	NM_001285751.1
<i>GR</i>	F: AGAGGGAGAGGAAATGGAG R: TTGAATGAGAAGGGTGGTC	121	XM_018050198.1
<i>PCK1</i>	F: GCGTTCAACGTCGGATTTCC R: CTCGATGCCGATCTTGGACA	105	XM_005688314.3
<i>PCK2</i>	F: TACGTGCTTCCGTTGAGCAT R: TTGGCCACAGAGTGAAGAC	177	XM_018054616.1
<i>PKA</i>	F: ATTGTCTCTGGGAAGGTGCG R: TCCACTTGTAGGAGTGTGCG	80	XM_018051193.1
<i>PRKAA2</i>	F: TTGATGATGAGGTGGTGGAG R: CCGTGAGAGAGCCAGAGAGT	138	XM_018044652.1
<i>PRKAB1</i>	F: CCACCACATCTCTCCAAGT R: GAGCACCATCACTCCATCCT	135	XM_013970630.2
<i>PGC1A</i>	F: CCGAGAATTCATGAGCAAT R: GATTGTGTGTTGGCCCTCTT	184	XM_018049155.1
<i>STK11</i>	F: GGACACCTTCTTGCTTCA R: CCCTTCCCGATGTTCTCAA	126	XM_018050463.1
<b>Circadian signaling</b>			
<i>BMAL1</i>	F: GCACGGCTTCTTCTTCTG R: TGCAGAAGCTTTTCGATCTGC	115	XM_018059578.1
<i>CLOCK</i>	F: GGGTAAAAGTCAAAGCCACCC R: ACCGGTTACTGAAAGCAGG	98	XM_018049467.1
<i>CRY1</i>	F: CTGGTCTTGGCAGTGGGAAA R: ACCAAAGCTCTCTCTTACCT	106	XM_013964057.2
<i>CRY2</i>	F: AAAGGGTTCCCTCTCGGTA R: TGCTTCATCCGCTCGATGTT	149	XM_018059193.1
<i>DBP</i>	F: GATACGGTGGAGGTGCTGAT R: TCCGAGGTCAAAGTCTCT	91	XM_018062728.1
<i>PER1</i>	F: ACCTGGAGAAGAGAAGTACCCT R: GAGGGCTGAGAAGTTTGACCA	96	XM_018064611.1
<i>PER2</i>	F: GACTGGTTAGGACGGGCTC R: CCACTGCTCATGTCCACGTC	150	XM_018040197.1
<b>mTOR signaling</b>			
<i>AKT1</i>	F: TTCAAGCCTCAGGTCACGTC R: ATGCTGTCGCTTGGTCAGG	101	NM_001285750.1
<i>mTOR</i>	F: CATCACCTTGCTCTCCGAA R: TACGGATCTCCTTGTGCTCG	116	NM_001285748.1
<i>RPTOR</i>	F: CATTAGTCCCAGACCAGACG R: CACCTCCATCTGCTTCTCGC	128	XM_018063632.1
<i>TSC1</i>	F: CAGACAACCCATGTTCCGA R: CAGAAGCCAGGTCGCCTAAA	107	NM_001314283.1
<i>TSC2</i>	F: GTCTCAACGAGAGCCCAAG R: GGGACTGCCGTGAAATTGGA	178	NM_001285553.1
<i>ACTG1</i>	F: ATGGCTACTGCTGCTGCT R: TTGAAGGTGGTCTCGTGGAT	161	XM_018063603.1

*ACACA* = acetyl-CoA carboxylase alpha; F = forward primer; R = reverse primer; *ACOX1* = acyl-CoA oxidase 1; *CPT1A* = carnitine palmitoyltransferase 1A; *CREB1* = cAMP-responsive element-binding protein 1; *CREBBP* = CREB-binding protein; *G6PDH* = glucose-6-phosphate dehydrogenase; *INSR* = insulin receptor; *NR1H3* = nuclear receptor subfamily 1 group H member 3; *GR* = glucocorticoid receptor; *PCK1* = phosphoenolpyruvate carboxykinase 1; *PCK2* = phosphoenolpyruvate carboxykinase 2, mitochondrial; *PKA* = protein kinase A; *PRKAA2* = protein kinase AMP-activated catalytic subunit alpha 2; *PRKAB1* = protein kinase AMP-activated noncatalytic subunit beta 1; *PGC1A* = peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *STK11* = serine-threonine kinase 11; *BMAL1* = aryl hydrocarbon receptor nuclear translocator-like protein 1; *CLOCK* = clock circadian regulator; *CRY1* = cryptochrome 1; *CRY2* = cryptochrome 2; *DBP* = D-box binding PAR bZIP transcription factor; *PER1* = period circadian regulator 1; *PER2* = period circadian regulator 2; *AKT1* = protein kinase B; *mTOR* = mammalian target of rapamycin; *RPTOR* = regulatory-associated protein of mTOR; *TSC1* = tuberous sclerosis 1; *TSC2* = tuberous sclerosis 2; *ACTG1* = actin gamma 1.

**Table 2**  
Effects of maternal intake restriction during midgestation on the blood biochemical indices of pregnant dams, fetuses and kids.

Item	Dams <sup>1</sup>				Fetuses <sup>1</sup>							Kids <sup>1</sup>								
	CON (n = 6)		RES (n = 6)		SEM	P-value	CON (n = 10)			RES (n = 10)				SEM	P-value	Diet			SEM	P-value
							Diet	Sex	Litter size	CON (n = 8)	RES (n = 8)	Diet	Sex			Litter size				
Hot carcass weight, kg	14.7 <sup>a</sup>	11.0 <sup>b</sup>	0.78	0.009	0.65	0.58	0.037	0.18	0.71	0.54	3.9 <sup>a</sup>	2.9 <sup>b</sup>	0.25	0.017	0.46	0.78				
NEFA, mmol/L	0.44 <sup>b</sup>	0.58 <sup>a</sup>	0.01	0.007	29.6 <sup>a</sup>	26.9 <sup>b</sup>	0.17	0.004	0.045	0.007	64.8	66.5	1.14	0.29	0.009	0.002				
TP, g/L	74.4 <sup>a</sup>	66.8 <sup>b</sup>	2.24	0.045	254.9	241.6	10.72	0.36	0.10	0.09	46.7	43.8	4.94	0.68	0.34	0.80				
T4, ng/mL	56.0	45.5	7.89	0.38	2.0	1.9	0.10	0.50	0.28	0.64	1.1	1.3	0.06	0.18	0.85	0.71				
T3, ng/mL	1.4	1.3	0.17	0.49	34.9	34.0	0.79	0.41	0.63	0.018	32.7	32.4	4.22	0.96	0.43	0.58				
Glucagon <sup>2</sup> , ng/L	24.4 <sup>b</sup>	54.0 <sup>a</sup>	4.45	0.002																

NEFA = nonesterified fatty acids; TP = total protein; T4 = thyroxine; T3 = triiodothyronine.

<sup>a, b</sup> Labeled means in a row without a common letter differ at  $P < 0.05$  for the effect of diet.

<sup>1</sup> Dams in the control (CON) group were provided 100% of their nutrient requirements. Dams in the restricted (RES) group were provided 60% of the intake of the CON group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

<sup>2</sup> Data were reported previously (Zhou et al., 2019b).

**Table 3**  
Effects of maternal intake restriction during midgestation on the blood amino acid profile of pregnant dams and postnatal kids (μmol/L).

Item	Dams <sup>1</sup>				Kids <sup>1</sup>								
	CON (n = 6)		RES (n = 6)		SEM	P-value	CON (n = 8)			RES (n = 8)			
							Diet	Sex	Litter size				
Asp	28.7	26.0	3.77	0.64	43.1	51.4	5.59	0.31	0.97	0.27			
Thr	54.6	63.3	5.44	0.29	82.7 <sup>b</sup>	113.2 <sup>a</sup>	7.10	0.011	0.46	0.069			
Ser	82.3 <sup>b</sup>	103.3 <sup>a</sup>	6.36	0.046	96.5	107.6	6.65	0.26	0.98	0.72			
Glu	108.0	126.4	9.76	0.22	171.7	177.3	10.28	0.70	0.20	0.20			
Gly	1,230.5	1,415.3	123.67	0.32	2,505.2	2,475.5	158.77	0.90	0.30	0.29			
Ala	643.1	652.9	61.76	0.91	646.4	620.6	34.19	0.59	0.43	0.42			
Val	287.7	243.7	26.24	0.27	429.7	467.5	27.00	0.33	0.49	0.021			
Cys	17.0	33.9	11.00	0.31	90.2	49.5	16.73	0.26	0.50	0.95			
Met	68.5	66.3	5.98	0.81	49.8	52.2	2.10	0.42	0.89	0.026			
Ile	125.8	129.8	7.23	0.71	159.4	181.1	12.56	0.24	0.97	0.49			
Leu	195.0	173.2	13.20	0.28	230.9	252.9	20.58	0.45	0.80	0.31			
Tyr	102.3	103.8	9.73	0.92	82.5	81.4	6.98	0.91	0.45	0.027			
Phe	77.1	68.7	7.76	0.47	72.0	75.5	5.00	0.62	0.96	0.09			
Lys	249.6	252.7	26.96	0.94	293.8	336.8	36.04	0.41	0.93	0.32			
His	70.2	71.4	8.95	0.93	85.5	74.3	5.88	0.20	0.36	0.81			
Arg	311.4	327.8	27.81	0.69	482.8	484.6	40.55	0.97	0.79	0.20			
EAA <sup>2</sup>	1,457.0	1,430.8	95.61	0.90	1,976.8	2,087.7	136.81	0.50	0.96	0.22			
NEAA <sup>2</sup>	2,194.8	2,427.7	159.40	0.37	3,545.4	3,513.7	165.98	0.90	0.56	0.45			
BCAA <sup>2</sup>	608.6	546.6	44.10	0.36	820.0	901.5	59.22	0.34	0.83	0.12			

<sup>a, b</sup> Labeled means in a row without a common letter differ at  $P < 0.05$  for the effect of diet.

<sup>1</sup> Dams in the control (CON) group were provided 100% of their nutrient requirements. Dams in the restricted (RES) group were provided 60% of the intake of the CON group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

<sup>2</sup> EAA refers to essential and conditionally essential, including Thr, Cys, Val, Met, Ile, Leu, Phe, Lys, His and Arg. NEAA refers to nonessential AA, including Asp, Ser, Glu, Gly, Ala and Tyr. BCAA refers to branched-chain AA, including Val, Ile and Leu.

affected ( $P < 0.05$ ) by litter size and were all higher ( $P < 0.05$ ) in the triplets than in the singletons and twins. The AA profile of the kids was not affected ( $P > 0.05$ ) by sex.

### 3.3. Glycogen and cAMP in the muscles of offspring

The cAMP and glycogen concentrations in the LT muscles of the offspring ( $P > 0.05$ , Table 4) were not altered by diet. The cAMP concentrations of the kids were affected ( $P = 0.006$ ) by litter size,

which was higher in the triplets than in the singleton and twins. The cAMP and glycogen levels of the fetuses were not influenced ( $P > 0.05$ ) by sex or litter size.

### 3.4. The mRNA and protein expression of the energy metabolism-related genes in the LT muscles

The mRNA expression of acyl-CoA oxidase 1 (ACOX1), nuclear receptor subfamily 1 group H member 3 (NR1H3), glucocorticoid

**Table 4**  
Effects of maternal intake restriction during midgestation on the cAMP and glycogen concentrations in the *Longissimus thoracis* of the offspring.

Item	Fetuses <sup>1</sup>				Kids <sup>1</sup>								
	CON (n = 10)		RES (n = 10)		SEM	P-value	CON (n = 8)			RES (n = 8)			
			Diet	Sex			Litter size						
cAMP, nmol/g protein	1.68	1.65	0.600	0.97	0.87	0.13	15.17	8.86	2.928	0.17	0.059	0.006	
Glycogen, mg/g protein	0.43	0.46	0.017	0.21	0.74	0.50	0.26	0.29	0.012	0.20	0.20	0.28	

<sup>1</sup> Dams in the control (CON) group were provided 100% of their nutrient requirements. Dams in the restricted (RES) group were provided 60% of the intake of the CON group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

receptor (*GR*) and protein kinase AMP-activated noncatalytic subunit beta 1 (*PRKAB1*) in the LT muscles of RES pregnant dams was upregulated ( $P < 0.05$ , Fig. 1A), whereas phosphoenolpyruvate carboxykinase 2 (*PCK2*) mRNA tended to be decreased ( $P = 0.075$ ).

The expression of AMPK $\alpha$  and STK11 proteins in the RES dams was not affected ( $P > 0.05$ , Fig. 1B). In the RES fetuses, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC1A*) mRNA was upregulated ( $P = 0.018$ , Fig. 2A), and *ACOX1* mRNA tended to be increased ( $P < 0.10$ ). The mRNA expression of *NR1H3* was downregulated in the RES kids ( $P < 0.05$ , Fig. 2B), but the *ACOX1* mRNA in the RES kids tended to be increased ( $P = 0.092$ ). The mRNA expression of these detected genes in the fetuses ( $P > 0.05$ ) was not affected by sex or litter size. The mRNA expression of *PCK1*

and *PRKAB1* in the kids was affected ( $P < 0.05$ ) by sex. The mRNA expression of *PCK1* in female kids was higher than that in male kids (0.81 vs. 2.29), but the mRNA expression of *PRKAB1* in female kids was lower than that in male kids (1.31 vs. 0.81). The mRNA expression of these genes was not altered ( $P > 0.05$ ) by litter size.

The p-STK11 protein abundance in the RES fetuses tended to be increased ( $P < 0.10$ ), and the ratio of p-STK11/STK11 in the RES fetuses and RES kids tended to be increased ( $P < 0.10$ , Fig. 2C and D). The p-AMPK $\alpha$  and AMPK $\alpha$  protein expression levels of the RES fetuses and RES kids were not affected ( $P > 0.05$ ). The p-AMPK $\alpha$ :AMPK $\alpha$  level in the fetuses ( $P = 0.028$ ) was affected by litter size and was lower in the triplets than in the singletons and twins. Sex did not affect the protein expression of these genes in the offspring ( $P > 0.05$ ).

The *CREB1*, CREB-binding protein (*CREBBP*) and *PKA* mRNA expression levels in the RES fetuses were reduced ( $P < 0.05$ , Fig. 3A). The expression levels of *CREBBP* and *PKA* mRNA were downregulated in the RES kids ( $P < 0.05$ , Fig. 3B), and the *CREB1* mRNA in the RES kids tended to be reduced ( $P = 0.099$ ). The expression of the p-*PKA* and *PKA* proteins in both the RES fetuses and RES kids was downregulated ( $P < 0.05$ , Fig. 3C and D). The p-*PKA* protein expression in the kids was affected ( $P = 0.019$ ) by litter size, which was lower in the singleton than in the twins and triplets. Protein expression was not affected ( $P > 0.05$ ) by sex in the offspring (Fig. 3C and D).

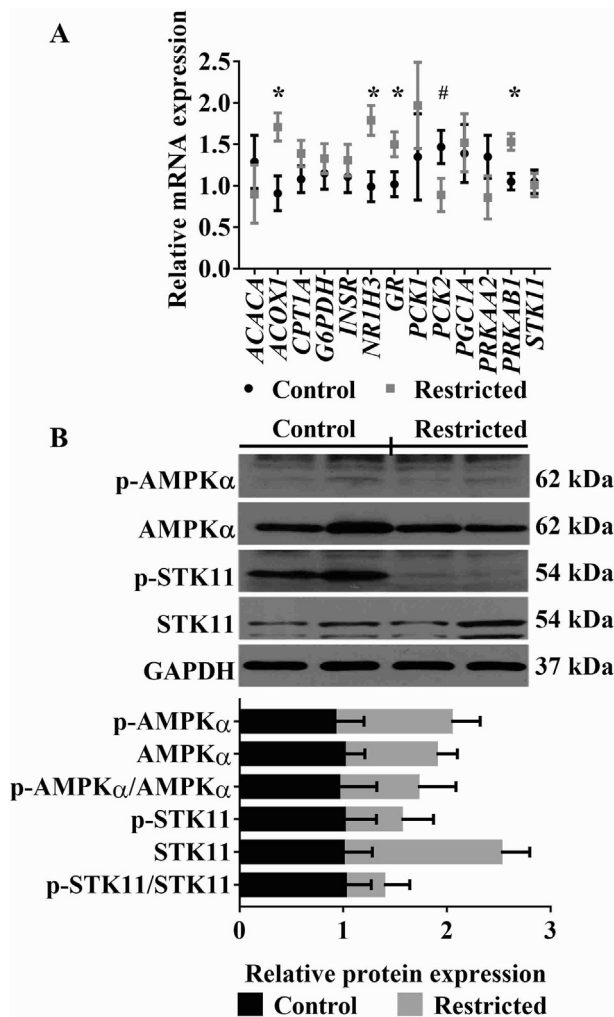
### 3.5. The mRNA expression of circadian CLOCK pathway genes in the LT muscles

In the RES group, fetal aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*) mRNA expression was reduced ( $P = 0.043$ , Table 5), and the fetal cryptochrome 2 (*CRY2*) mRNA level tended to be reduced ( $P = 0.051$ ), but the fetal D-box binding PAR bZIP transcription factor (*DBP*) mRNA level tended to be increased ( $P = 0.068$ ). *DBP* mRNA expression in the RES kids was decreased ( $P = 0.049$ ), but *CLOCK* mRNA expression was increased ( $P = 0.019$ ). *BMAL1* mRNA expression in the kids was affected ( $P = 0.023$ ) by litter size and was higher in triplets than in twins. The influence of sex on the mRNA expression of these circadian genes in the fetuses and kids was not found ( $P > 0.05$ ).

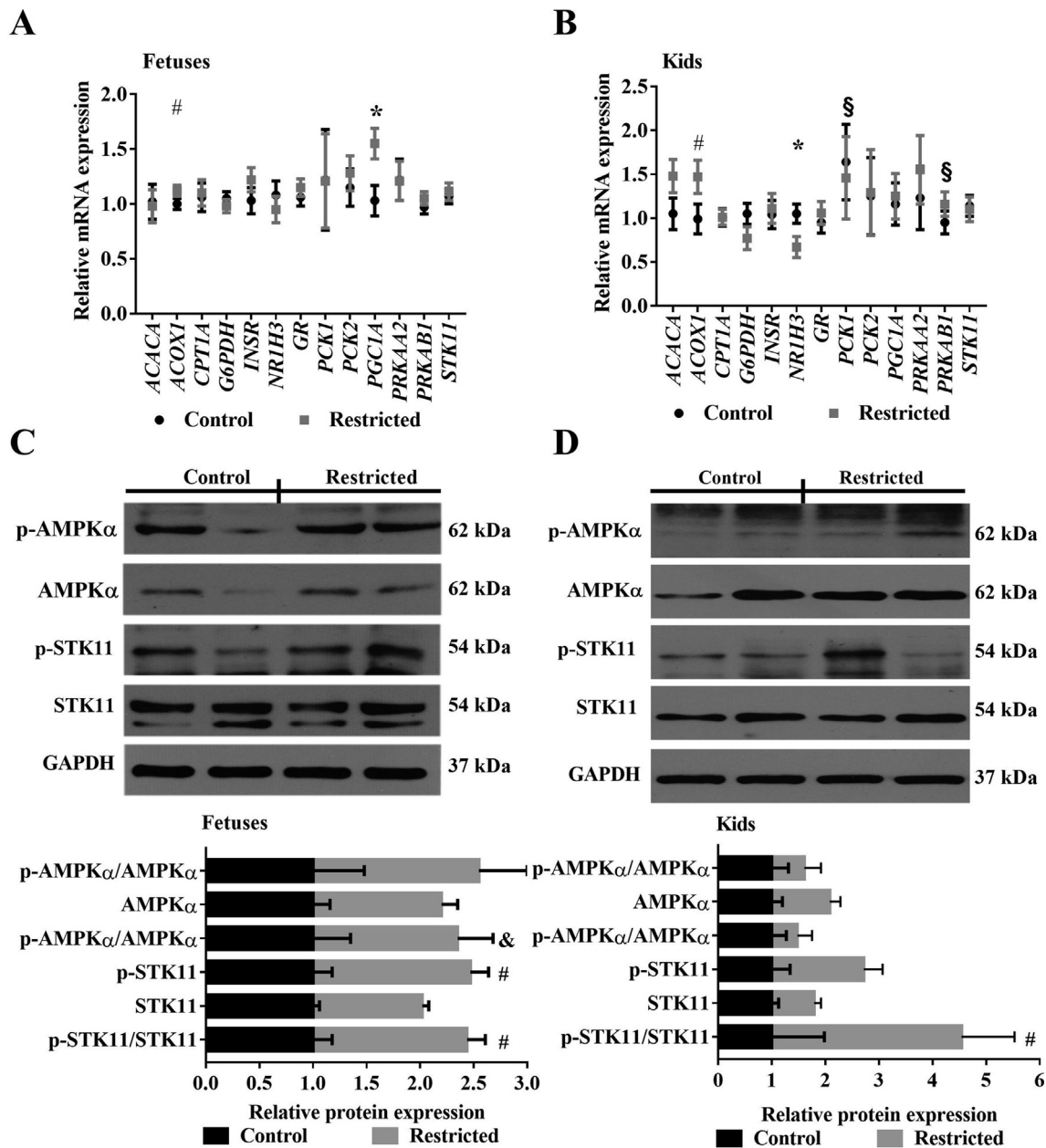
### 3.6. The mRNA and protein expression of the mTOR pathway

In the RES groups, protein kinase B (*AKT1*), *mTOR*, and regulatory-associated protein of mTOR (*RPTOR*) mRNA expression in the LT muscles of the fetuses was downregulated ( $P < 0.05$ , Fig. 4A), and fetal *TSC2* mRNA expression was upregulated ( $P = 0.049$ ). The mRNA expression of *TSC2* in the RES kids was upregulated ( $P = 0.01$ , Fig. 4B), and the mRNA expression of *TSC1* tended to be increased ( $P = 0.069$ ). The mRNA expression of the above genes in the fetuses and kids was not affected ( $P > 0.05$ ) by sex or litter size.

The protein expression of mTOR and p-mTOR in both the LT muscles of the RES fetuses and RES kids was reduced ( $P < 0.05$ , Fig. 4C and D). The protein expression of p-TSC2 and the ratio of p-TSC2/TSC2 in the RES kids tended to be upregulated ( $P < 0.10$ ), while the ratio of p-mTOR/mTOR tended to be decreased ( $P = 0.095$ ). Fetal p-mTOR protein expression was affected ( $P = 0.019$ ) by sex and was lower in females than in males. mTOR and p-mTOR/mTOR expression in the fetuses was affected ( $P < 0.05$ ) by litter size. The fetal mTOR level in the triplets was higher ( $P = 0.017$ ) than those in the singleton and twins, but the fetal p-mTOR/mTOR level in the singleton was higher ( $P = 0.014$ ) than those in the twins and triplets. mTOR protein expression in the kids was affected ( $P = 0.046$ ) by sex and was higher in the female kids than in the males. The



**Fig. 1.** The relative mRNA (A) and protein expression (B) of energy metabolism-related genes in the *Longissimus thoracis* muscles of the dams ( $n = 6$ ) at 100 d of gestation. *ACACA* = acetyl-CoA carboxylase alpha; *ACOX1* = acyl-CoA oxidase 1; *CPT1A* = carnitine palmitoyltransferase 1A; *G6PDH* = glucose-6-phosphate dehydrogenase; *INSR* = insulin receptor; *NR1H3* = nuclear receptor subfamily 1 group H member 3; *GR* = glucocorticoid receptor; *PCK1* = phosphoenolpyruvate carboxykinase 1; *PCK2* = phosphoenolpyruvate carboxykinase 2, mitochondrial; *PGC1A* = peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *PRKAA2* = protein kinase AMP-activated catalytic subunit alpha 2; *PRKAB1* = protein kinase AMP-activated noncatalytic subunit beta 1; *STK11* = serine-threonine kinase 11; p-AMPK $\alpha$  = phospho-AMP-activated protein kinase; p-STK11 = phospho-STK11; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. \* $P < 0.05$  and # $0.05 \leq P < 0.10$  for the effect of diet. Dams in the control group were provided 100% of their nutrient requirements. Dams in the restricted group were provided 60% of the intake of the control group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.



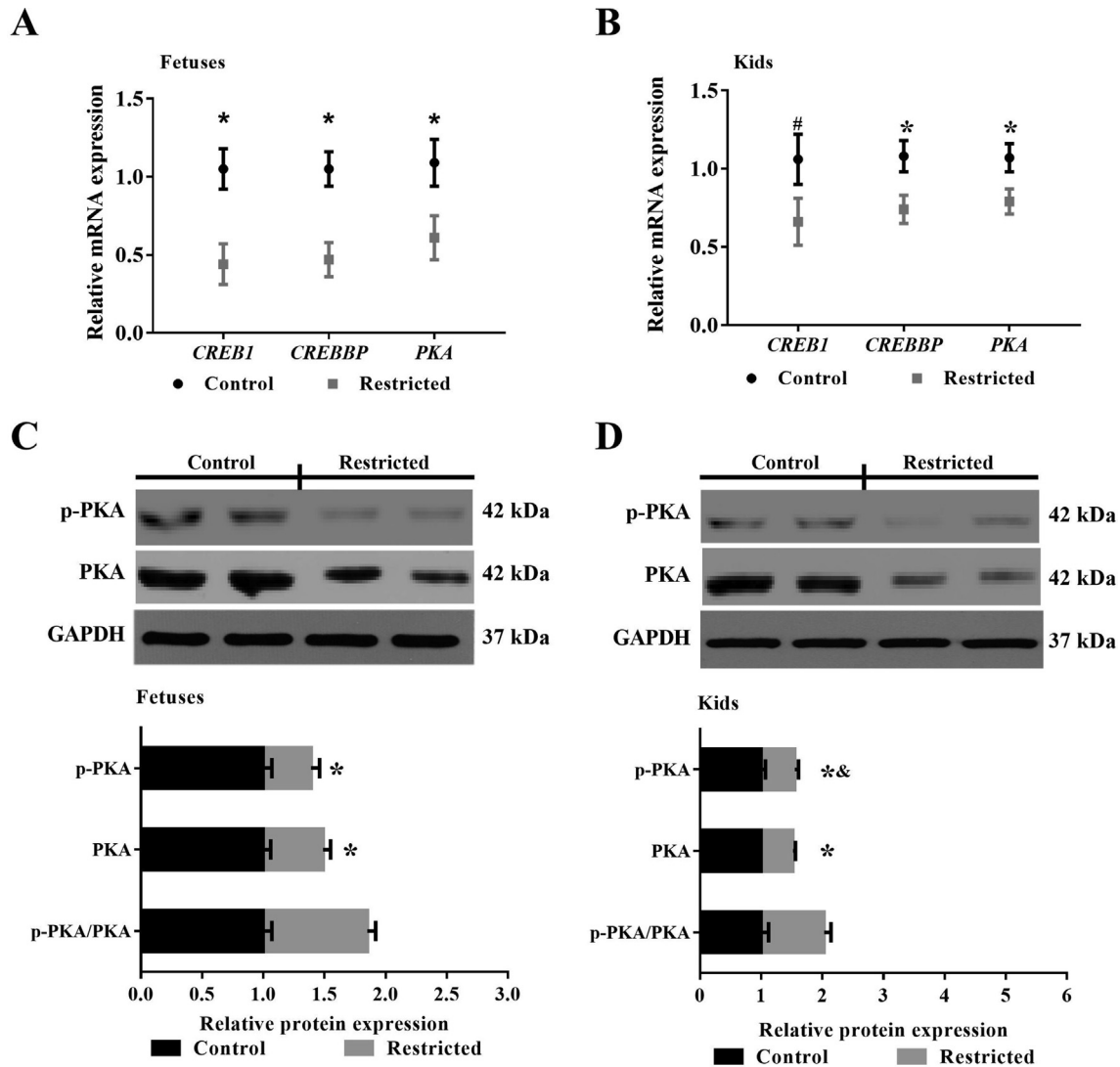
**Fig. 2.** The relative expression of energy metabolism-related genes in the *Longissimus thoracis* muscles of the offspring. mRNA expression in the fetuses (A,  $n = 10$ ) and in the kids (B,  $n = 8$ ). Protein expression in the fetuses (C) and in the kids (D). ACACA = acetyl-CoA carboxylase alpha; ACOX1 = acyl-CoA oxidase 1; CPT1A = carnitine palmitoyltransferase 1A; G6PDH = glucose-6-phosphate dehydrogenase; INSR = insulin receptor; NRIH3 = nuclear receptor subfamily 1 group H member 3; GR = glucocorticoid receptor; PCK1 = phosphoenolpyruvate carboxykinase 1; PCK2 = phosphoenolpyruvate carboxykinase 2, mitochondrial; PGC1A = peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA2 = protein kinase AMP-activated catalytic subunit alpha 2; PRKAB1 = protein kinase AMP-activated noncatalytic subunit beta 1; STK11 = serine-threonine kinase 11; p-AMPKα = phospho-AMP-activated protein kinase; p-STK11 = phospho-STK11; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. \* $P < 0.05$  and # $0.05 \leq P < 0.10$  for the effect of diet; § $P < 0.05$  for the effect of sex; & $P < 0.05$  for the effect of litter size. Dams in the control group were provided 100% of their nutrient requirements. Dams in the restricted group were provided 60% of the intake of the control group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

expression of mTOR and p-mTOR was also affected ( $P < 0.05$ ) by litter size, with both expression levels lower in the triplets than in the singletons but intermediate in the twins.

#### 4. Discussion

Food deprivation in humans and mammals is common in underdeveloped areas, and nutrient restriction during gestation programs muscle development and metabolism in offspring (Beauchamp and Harper, 2015; Brown and Hay, 2016); however, the

mechanism of this effect remains unclear. In the present study, the 40% intake restriction reduced the carcass weight of dams and kids and reduced the plasma TP contents of dams and fetuses. Maternal malnutrition did not affect the cAMP and glycogen contents of the offspring muscles, whereas the mRNA expression of genes associated with fatty acid oxidation and regulation in the mother and offspring was upregulated, and the mRNA and protein expression of genes involved in the mTOR pathway in offspring were downregulated. Moreover, the mRNA expression of the CLOCK pathway was affected in offspring.



**Fig. 3.** The relative expression of PKA and CREB genes in the *Longissimus thoracis* muscles of the fetuses at 100 d of gestation and kids at 90 d after birth. mRNA expression in the fetuses (A,  $n = 10$ ) and in the kids (B,  $n = 8$ ). Protein expression in the fetuses (C) and in the kids (D). PKA = protein kinase A; CREB1 = cAMP-responsive element-binding protein 1; CREBBP = CREB-binding protein; p-PKA = phospho-PKA; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. \* $P < 0.05$  and # $0.05 \leq P < 0.10$  for the effect of diet;  $^{\&}P < 0.05$  for the effect of litter size. Dams in the control group were provided 100% of their nutrient requirements. Dams in the restricted group were provided 60% of the intake of the control group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

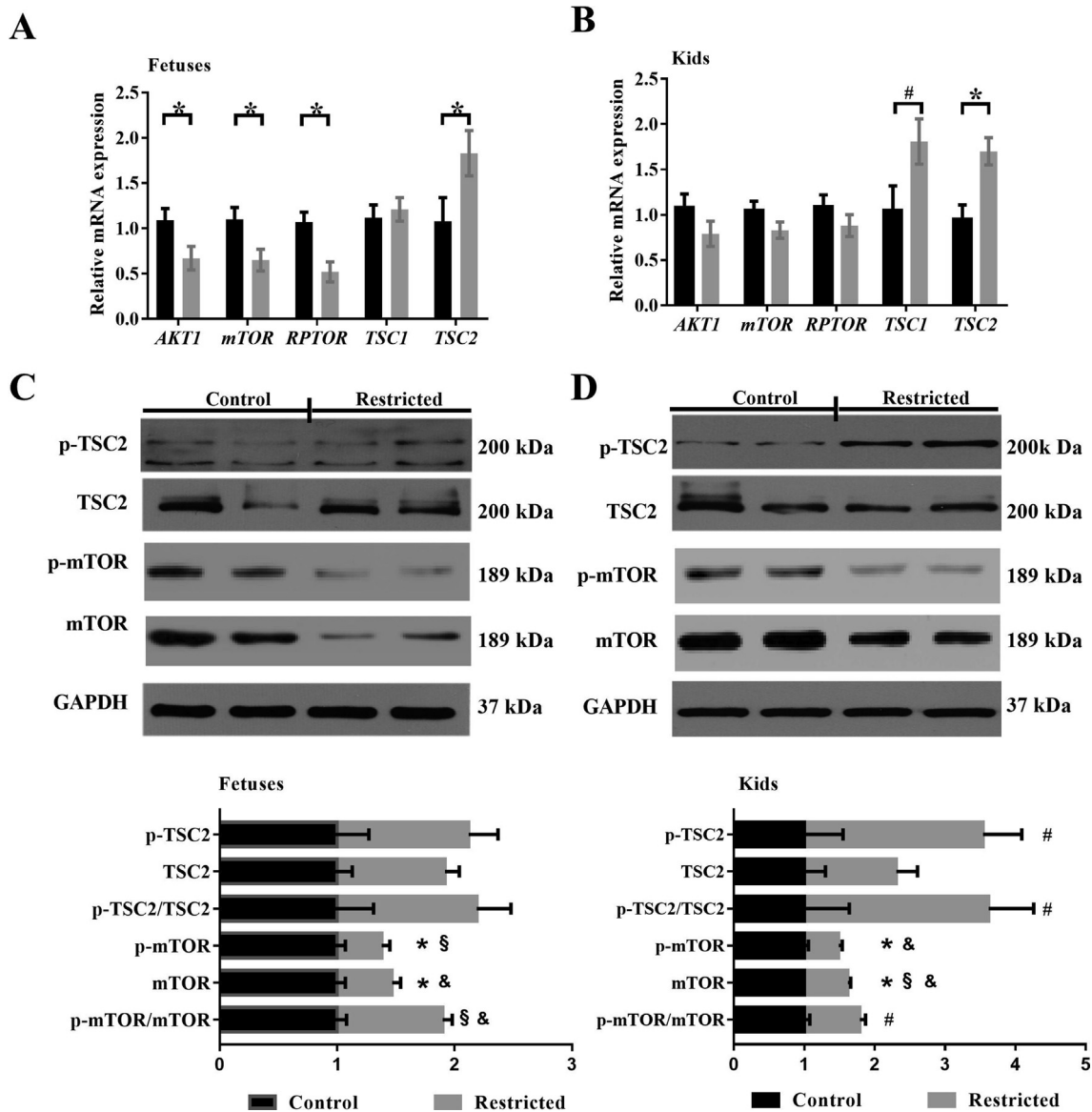
Intake restriction reduced the hot carcass weight of the dams, indicating that the maternal muscle mass declined. The TP concentrations in the maternal and fetal plasma were reduced, whereas the venous AA profile of dams and kids, the AA profile in the umbilical cord blood of fetal goats that was reported previously (Chen et al., 2019), and the glucose and insulin concentrations that we have reported (Zhou et al., 2019b) were not affected. These results suggest an insufficient supply of protein in the dams and fetuses. However, 40% maternal restriction did not reduce the weight and birth weight of the fetuses, but the body weight and carcass weight of the kids after birth were reduced (Zhou et al., 2019a). These results suggest in utero maternal compensation and defective muscle growth and development programming after birth.

ACOX1 is the first rate-limiting enzyme of fatty acid  $\beta$ -oxidation, which catalyzes acyl-CoA into 2-trans-enoyl-CoA. NR1H3 (also known as LXR $\alpha$ ) regulates fat homeostasis and plays an important role in cholesterol metabolism and lipid synthesis (Beitowski, 2008; Cha and Repa, 2007). Glucocorticoid receptor (GR) is

associated with the stress response caused by a high level of cortisol under starvation, which is a common target regulator of intrauterine metabolic programming (Chadio et al., 2017). PRKAB1 is a regulatory subunit in the AMPK protein complex that monitors cellular energy status (Lin and Hardie, 2018). The increase in the blood NEFA concentration and the ACOX1, NR1H3, GR and PRKAB1 mRNA expression in the restricted dams indicated the change in lipid metabolism and the upregulation of fatty acid oxidation in the LT muscle. PCK2 in muscle tissues catalyzes the conversion of oxaloacetate into phosphoenolpyruvate, by which the oxaloacetate availability of the tricarboxylic acid (TCA) cycle and glucose homeostasis are regulated (Stark and Kibbey, 2014). Downregulation of PCK2 mRNA in muscle implied reduced glucose availability in the muscle tissue and downregulated TCA cycle oxidation–energy pathways. Therefore, intake restriction probably resulted in the stimulation of fatty acid oxidation and the suppression of glucose oxidative degradation in the LT muscles of the dams.

As one of the target organs for intrauterine metabolic programming (Beauchamp and Harper, 2015; Sharples et al., 2016),





**Fig. 4.** The relative expression of mTOR signaling genes in the *Longissimus thoracis* muscles of the fetuses at 100 d of gestation and kids at 90 d after birth. mRNA expression in the fetuses (A,  $n = 10$ ) and in the kids (B,  $n = 8$ ). Protein expression in the fetuses (C) and in the kids (D). *AKT1* = protein kinase B; *mTOR* = mammalian target of rapamycin; *RPTOR* = regulatory-associated protein of mTOR; *TSC1* = tuberous sclerosis 1; *TSC2* = tuberous sclerosis 2; p-TSC2 = phospho-TSC2; p-mTOR = phospho-mTOR; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. \* $P < 0.05$  and # $0.05 \leq P < 0.10$  for the effect of diet;  $^{\text{§}}P < 0.05$  for the effect of sex;  $^{\text{&}}P < 0.05$  for the effect of litter size. Dams in the control group were provided 100% of their nutrient requirements. Dams in the restricted group were provided 60% of the intake of the control group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

**Table 5**  
Effects of maternal intake restriction during midgestation on the mRNA expression of circadian genes in the *Longissimus thoracis* muscles of offspring.

Item	Fetuses <sup>1</sup>		SEM	P-value			Kids <sup>1</sup>		SEM	P-value		
	CON ( $n = 10$ )	RES ( $n = 10$ )		Diet	Sex	Litter size	CON ( $n = 8$ )	RES ( $n = 8$ )		Diet	Sex	Litter size
<i>DBP</i>	1.11	1.51	0.146	0.068	0.33	0.084	1.08 <sup>a</sup>	0.65 <sup>b</sup>	0.137	0.049	0.74	0.58
<i>BMAL1</i>	1.16 <sup>a</sup>	0.66 <sup>b</sup>	0.164	0.043	0.21	0.67	1.20	0.86	0.160	0.17	0.53	0.023
<i>CLOCK</i>	1.16	1.11	0.210	0.86	0.66	0.24	0.95 <sup>b</sup>	4.55 <sup>a</sup>	0.863	0.019	0.077	0.21
<i>CRY1</i>	1.11	1.48	0.416	0.52	0.31	0.061	1.15	0.98	0.110	0.31	0.53	0.051
<i>CRY2</i>	1.13	0.70	0.147	0.051	0.60	0.57	1.06	1.03	0.174	0.90	0.92	0.92
<i>PER1</i>	1.19	1.50	0.218	0.32	0.34	0.76	1.02	1.53	0.194	0.10	0.21	0.74
<i>PER2</i>	1.09	0.66	0.186	0.11	0.87	0.53	1.11	0.82	0.183	0.28	0.66	0.87

*DBP* = D-box binding PAR bZIP transcription factor; *BMAL1* = aryl hydrocarbon receptor nuclear translocator-like protein 1; *CLOCK* = clock circadian regulator; *CRY1* = cryptochrome 1; *CRY2* = cryptochrome 2; *PER1* = period circadian regulator 1; *PER2* = period circadian regulator 2.

<sup>a, b</sup> Labeled means in a row without a common letter differ at  $P < 0.05$  for the effect of diet.

<sup>1</sup> Dams in the control (CON) group were provided 100% of their nutrient requirements. Dams in the restricted (RES) group were provided 60% of the intake of the CON group during 45 to 100 d of gestation and then realimented to 100% of nutrient requirements.

muscle tissue is involved in blood glucose uptake and clearance and the regulation of glucose and lipid metabolism homeostasis. In our study, maternal restriction did not alter the cAMP and glycogen concentrations of LT muscles in the offspring. The mRNA expression of glucose-6-phosphate dehydrogenase (*G6PDH*), *PCK1*, *PRKAA2* and *PRAKB1* and the protein expression of AMPK $\alpha$  in the offspring were also unaffected in our study. These results were in line with the findings for lambs after 50% intrauterine restriction during early to midgestation (Zhu et al., 2004). We speculated that 40% maternal intake restriction during midgestation did not alter the glucose storage capacity and consumption in the LT muscles of the fetuses and kids. However, maternal intake restriction increased the mRNA expression of *ACOX1* in the LT muscles of the fetuses and kids, and these results were consistent with the mRNA expression of *ACOX1* in the dams. Moreover, the mRNA expression of *PGC1A* in the fetuses and the protein expression of p-STK11/STK11 in the fetuses and kids were upregulated, but *NR1H3* mRNA expression was downregulated in the kids. *PGC1A* promotes mitochondrial oxidative metabolism (Supruniuk et al., 2017), while p-STK11 stimulates the phosphorylation of *ACOX1* (Willows et al., 2017) and inhibits fat synthesis. The increase in *ACOX1* and *PGC1A* mRNA and p-STK11/STK11 protein in restricted fetuses and kids indicate that maternal restriction upregulated lipolysis of muscle tissue and fatty acid  $\beta$ -oxidation as an energy source. Similarly, 40% maternal feed restriction also upregulated the mRNA expression of *PGC1A* in the LT muscles of fetal calves during mid-to late gestation (Paradis et al., 2017).

Maternal restriction simultaneously reduced the mRNA expression of *CREB1* and *CREBBP* in both the fetuses and kids. Recent studies have found that CREB interacts with *PGC1A*, *NR1H3* and *DBP* to regulate glucose synthesis, gluconeogenesis (Kim et al., 2017), and fat metabolism (Zheng et al., 2016) in mouse livers. CREB also alters the main clock in the supraoptic nucleus of mice by phosphorylation at Ser133 (Wheaton et al., 2018), while CREB expression is regulated by rhythm clock genes to maintain metabolic rhythms. A study in mouse muscle revealed that knockout of the rhythmic gene *BMAL1* leads to disturbances in the transcription of glucose-, fat- and protein metabolism-related genes (Dyar et al., 2018). Consistent with the change in the CREB and *CREBBP* regulators in the offspring of this study, maternal restriction had a tendency to increase *DBP* mRNA in the fetal muscle, and *DBP* mRNA expression was decreased in the kids. Moreover, maternal restriction reduced the mRNA expression of *BMAL1* and *CRY2* in the fetuses, and the mRNA expression of *CLOCK* was increased in the kids. In the rhythmic *CLOCK* signaling pathway, *DBP* activates the transcription of *PER*, while period circadian regulator (*PER*) inhibits the expression of *CLOCK* and *BMAL1* by forming a dimer with *CRY* (Hamilton and Kay, 2008). The mRNA expression patterns of *DBP*, *BMAL1* and *CLOCK* in the fetuses and kids in this study were consistent with the *CLOCK* signaling transmission pathway. Of note, *CLOCK* plays a role in histone acetylation (Grimaldi et al., 2009), and this epigenetic mechanism is closely related to developmental programming. We proposed that maternal intake restriction programmed the CREB-CREBBP regulatory factors to alter the mRNA expression of energy metabolism and the *CLOCK* pathway in the offspring.

The AKT-TSC-mTORC1 signaling pathway is one of the main regulators of muscle protein synthesis, which occurs in response to cellular energy, AA, insulin and insulin-like growth factor 1 (IGF1) signals (Hart et al., 2019; Luo et al., 2018). Maternal restriction downregulated the mRNA expression of *AKT1*, *mTOR* and *RPTOR* and the protein expression of mTOR and p-mTOR in the LT muscles of fetuses, but the mRNA expression of *TSC2* was upregulated. The

expression of *TSC1* and *TSC2* mRNA and p-TSC2 protein in the LT muscles of kids was upregulated by maternal restriction, whereas the protein expression of mTOR and p-mTOR was downregulated. These results showed that maternal restriction altered mTOR pathway signaling in both the fetuses and kids. Similarly, a decrease in the p-mTOR protein of the fetal LT muscle was observed under 50% maternal restriction during early to midgestation (Zhu et al., 2004). Although the TP level was decreased, the blood glucose, insulin, IGF1 (Zhou et al., 2019b), and muscular glycogen levels in the offspring of the present study were unaffected. The reason may be ascribed to the reduction in the overall protein or AA supply under intake restriction when energy is deficient. A previous study in intrauterine growth restricted (IUGR) sheep showed that AA perfusion reduces the rate of protein degradation and increases protein deposition by 150%, and the AA level independently regulates mTOR pathway signaling (Brown et al., 2012). In addition to energy, 40% maternal intake restriction may aggravate the lack of protein and AA, leading to the downregulation of the mTOR pathway in offspring, which affects muscle tissue protein synthesis and muscle mass. Moreover, recent studies found that rhythmic per protein regulates the mTORC1 signaling pathway by recruiting *TSC1* in the mouse (Wu et al., 2019), and the rhythmic factor *BMAL1* is also tightly linked to mTOR pathway protein synthesis via *S6K* (Khapre et al., 2014). These findings are consistent with the change in the *CLOCK* and mTOR pathways in this study, and these results suggest the connection among intrauterine malnutrition, rhythm disruption, and protein synthesis in the skeletal muscles of the offspring.

Furthermore, both the mRNA and protein expression levels of PKA were decreased in the offspring from the restricted group in this study. CREB is located downstream of the PKA factor (Delghandi et al., 2005), and PKA phosphorylates raptor to regulate mTORC1 (Jewell et al., 2019). It is reasonable to conclude that maternal intake restriction alters the PKA-CREB pathway to regulate energy metabolism, *CLOCK* signaling and protein synthesis and leads to metabolic programming in the LT muscles of the offspring. The classical pathway for the regulation of glucose metabolism under energy-deficient conditions is the glucagon-cAMP-PKA pathway (Goldstein and Hager, 2018). Elevated glucagon caused by intake restriction in dams was observed, but the cAMP and glycogen concentrations in the LT muscles of offspring were not altered. Intermediate mediators between the high level of maternal glucagon and the downstream PKA-CREB pathway need to be identified.

The effects of sex and litter size on the phenotype and metabolism of the skeletal muscles of mammals have been reported (Dearden et al., 2018; Symonds and Budge, 2009; Tsiplakou et al., 2016; Zhong et al., 2013). In this study, the effects of litter size on blood and tissue metabolites and gene expression in offspring were also observed, such as TP, glucagon, Val, Met, Tyr and cAMP concentrations and mTOR protein expression. Intake restriction of dams apparently leads to a more severe protein deficiency in triplets than in singletons and twins. Sex also influenced the blood TP concentration and mTOR (or p-mTOR) protein expression in offspring. The effects of sex and litter size on protein metabolism in IUGR offspring need further investigation.

## 5. Conclusions

Using the goat model in this study, maternal 60% energy-protein undernutrition consistently upregulated the expression of genes involved in PKA-CREB and mTOR signals, downregulated *ACOX1* mRNA expression, and affected the *CLOCK* pathway in the LT

muscles of nutrition-restricted fetuses and kids. We inferred that maternal intake restriction during midgestation programmed the PKA-CREB pathway in the skeletal muscle of offspring to upregulate fat oxidation, downregulate protein synthesis, and alter the circadian clock. These results reveal the role of the PKA-CREB pathway in metabolic programming in the skeletal muscles of offspring exposed to intrauterine malnutrition and thus deepen our understanding of the molecular mechanism of metabolic adaptation in skeletal muscle caused by maternal undernutrition in humans and mammals.

### Author contributions

**Xiaoling Zhou:** Investigation, Data curation, Writing; **Qiong-xian Yan:** Project administration, Conceptualization; **Hong Yang:** Investigation, Data curation; **Ao Ren:** Methodology; **Zhixiong He:** Visualization, Formal analysis; **Zhiliang Tan:** Conceptualization, Funding acquisition.

### Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

### Acknowledgments

This work was supported by the National Natural Science Foundation of China [31730092, 31760678 and 31402105]; and Hunan innovative Province construction project [2019RS3021]. The authors thanked Shaoxun Tang for the instruction for data analysis and Xuefeng Han for the assistance in Western blotting detection.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2021.09.006>.

### References

- Batool R, Butt MS, Sultan MT, Saeed F, Naz R. Protein–energy malnutrition: a risk factor for various ailments. *Crit Rev Food Sci Nutr* 2015;55:242–53.
- Beauchamp B, Harper ME. In utero undernutrition programs skeletal and cardiac muscle metabolism. *Front Physiol* 2015;6:401.
- Beitowsky J. Liver X receptors (LXR) as therapeutic targets in dyslipidemia. *Cardiovasc Ther* 2008;26:297–316.
- Boyle KE, Patinkin ZW, Shapiro ALB, Bader C, Vanderlinden L, Kechris K, et al. Maternal obesity alters fatty acid oxidation, AMPK activity, and associated DNA methylation in mesenchymal stem cells from human infants. *Mol Metab* 2017;6:1503–16.
- Brown LD, Hay Jr WW. Impact of placental insufficiency on fetal skeletal muscle growth. *Mol Cell Endocrinol* 2016;435:69–77.
- Brown LD, Rozance PJ, Thorn SR, Friedman JE, Hay Jr WW. Acute supplementation of amino acids increases net protein accretion in IUGR fetal sheep. *Am J Physiol Endocrinol Metab* 2012;303:E352–64.
- Cha J-Y, Repa JJ. The liver X receptor (LXR) and hepatic lipogenesis: the carbohydrate-response element-binding protein is a target gene of LXR. *J Biol Chem* 2007;282:743–51.
- Chadio S, Kotsampasi B, Taka S, Liandris E, Papadopoulos N, Plakokefalos E. Epigenetic changes of hepatic glucocorticoid receptor in sheep male offspring undernourished in utero. *Reprod Fertil Dev* 2017;29:1995–2004.
- Chen W, Chen Z, Tang B, Xiao F. Management and feeding techniques of multi-foetus dam-A case study of Liuyang black goat. *China Livestock Poultry Breed Indust* 2021;17:123–4.
- Chen W, Yan Q, Yang H, Zhou X, Tan Z. Effects of restrictions on maternal feed intake on the immune indexes of umbilical cord blood and liver Toll-like receptor signaling pathways in fetal goats during pregnancy. *J Anim Sci Biotechnol* 2019;10:29.

- Cui X, Yao L, Yang X, Gao Y, Fang F, Zhang J, et al. SIRT6 regulates metabolic homeostasis in skeletal muscle through activation of AMPK. *Am J Physiol Endocrinol Metab* 2017;313:E493–505.
- Dearden L, Bouret SG, Ozanne SE. Sex and gender differences in developmental programming of metabolism. *Mol Metab* 2018;15:8–19.
- Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal* 2005;17:1343–51.
- Dyar KA, Hubert MJ, Mir AA, Ciciliot S, Lutter D, Greulich F, et al. Transcriptional programming of lipid and amino acid metabolism by the skeletal muscle circadian clock. *PLoS Biol* 2018;16:e2005886.
- Eckel-Mahan KL, Patel VR, Mohney RP, Vignola KS, Baldi P, Sassone-Corsi P. Coordination of the transcriptome and metabolome by the circadian clock. *Proc Natl Acad Sci U S A* 2012;109:5541–6.
- Goldstein I, Hager GL. The three Ds of transcription activation by glucagon: direct, delayed, and dynamic. *Endocrinology* 2018;159:206–16.
- Gonzalez JM, Camacho LE, Ebarb SM, Swanson KC, Vonnahme KA, Stelzleni AM, et al. Realimentation of nutrient restricted pregnant beef cows supports compensatory fetal muscle growth. *J Anim Sci* 2013;91:4797–806.
- Grimaldi B, Nakahata Y, Kaluzova M, Masubuchi S, Sassone-Corsi P. Chromatin remodeling, metabolism and circadian clocks: the interplay of CLOCK and SIRT1. *Int J Biochem Cell Biol* 2009;41:81–6.
- Hales CN, Barker DJ. The thrifty phenotype hypothesis. *Br Med Bull* 2001;60:5–20.
- Hamilton EE, Kay SA. SnapShot: circadian clock proteins. *Cell* 2008;135:368–e1.
- Hardie DG. Sensing of energy and nutrients by AMP-activated protein kinase. *Am J Clin Nutr* 2011;93:891S–6S.
- Hart B, Morgan E, Alejandro E. Nutrient sensor signaling pathways and cellular stress in fetal growth restriction. *J Mol Endocrinol* 2019;62:R155–65.
- He ZX, Sun ZH, Beauchemin KA, Yang WZ, Tang SX, Zhou CS, et al. Effect of protein or energy restriction during late gestation on hormonal and metabolic status in pregnant goats and postnatal male offspring. *Animal* 2015;9:1843–51.
- Hochberg Z, Feil R, Constanca M, Fraga M, Junien C, Carel JC, et al. Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* 2011;32:159–224.
- Jewell JL, Fu V, Hong AW, Yu F-X, Meng D, Melick CH, et al. GPCR signaling inhibits mTORC1 via PKA phosphorylation of Raptor. *eLife* 2019;8.
- Khapre RV, Kondratova AA, Patel S, Dubrovsky Y, Wrobel M, Antoch MP, et al. BMAL1-dependent regulation of the mTOR signaling pathway delays aging. *Aging* 2014;6:48–57.
- Kim H, Zheng Z, Walker PD, Kapatos G, Zhang K. CREBH maintains circadian glucose homeostasis by regulating hepatic glycogenolysis and gluconeogenesis. *Mol Cell Biol* 2017;37:e00048-17.
- Lie S, Morrison JL, Williams-Wyss O, Suter CM, Humphreys DT, Ozanne SE, et al. Impact of periconceptional and preimplantation undernutrition on factors regulating myogenesis and protein synthesis in muscle of singleton and twin fetal sheep. *Phys Rep* 2015;3:e12495.
- Lin SC, Hardie DG. AMPK: sensing glucose as well as cellular energy status. *Cell Metabol* 2018;27:299–313.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– $\Delta\Delta CT$  method. *Methods* 2001;25:402–8.
- Luo Y, Xu W, Li G, Cui W. Weighing in on mTOR complex 2 signaling: the expanding role in cell metabolism. *Oxid Med Cell Longev* 2018;2018:7838647.
- Mendonca JE, Vilela MC, Bittencourt H, Lapa RM, Oliveira FG, Alessio ML, et al. GFAP expression in astrocytes of suprachiasmatic nucleus and medial preoptic area are differentially affected by malnutrition during rat brain development. *Nutr Neurosci* 2004;7:223–34.
- Mohrhauser DA, Taylor AR, Gonda MG, Underwood KR, Pritchard RH, Wertz-Lutz AE, et al. The influence of maternal energy status during mid-gestation on beef offspring tenderness, muscle characteristics, and gene expression. *Meat Sci* 2015;110:201–11.
- Ozanne SE. Metabolic programming—knowns, unknowns and possibilities. *Nat Rev Endocrinol* 2014;11:67–8.
- Paradis F, Wood KM, Swanson KC, Miller SP, McBride BW, Fitzsimmons C. Maternal nutrient restriction in mid-to-late gestation influences fetal mRNA expression in muscle tissues in beef cattle. *BMC Genom* 2017;18:632.
- Reinke H, Asher G. Crosstalk between metabolism and circadian clocks. *Nat Rev Mol Cell Biol* 2019;20:227–41.
- Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell* 2017;168:960–76.
- Sen U, Sirin E, Ensoy U, Aksoy Y, Ulutas Z, Kuran M. The effect of maternal nutrition level during mid-gestation on postnatal muscle fibre composition and meat quality in lambs. *Anim Prod Sci* 2016;56:834–43.
- Sharples AP, Stewart CE, Seaborne RA. Does skeletal muscle have an 'epi'-memory? The role of epigenetics in nutritional programming, metabolic disease, aging and exercise. *Aging Cell* 2016;15:603–16.
- Stark R, Kibbey RG. The mitochondrial isoform of phosphoenolpyruvate carboxykinase (PEPCK-M) and glucose homeostasis: has it been overlooked? *Biochim Biophys Acta* 2014;1840:1313–30.
- Stenvers DJ, Scheer FAJL, Schrauwen P, la Fleur SE, Kalsbeek A. Circadian clocks and insulin resistance. *Nat Rev Endocrinol* 2019;15:75–89.
- Supruniuk E, Miktosz A, Chabowski A. The implication of PGC-1 $\alpha$  on fatty acid transport across plasma and mitochondrial membranes in the insulin sensitive tissues. *Front Physiol* 2017;8:923.

- Symonds ME, Budge H. Nutritional models of the developmental programming of adult health and disease: workshop on 'Nutritional models of the developmental origins of adult health and disease'. *Proc Nutr Soc* 2009;68:173–8.
- Tsiplakou E, Papadomichelakis G, Sparaggis D, Sotirakoglou K, Georgiadou M, Zervas G. The effect of maternal or artificial milk, age and sex on three muscles fatty acid profile of Damascus breed goat kids. *Livest Sci* 2016;188:142–52.
- Vilela MC, Mendonca JE, Bittencourt H, Lapa RM, Alessio ML, Costa MS, et al. Differential vulnerability of the rat retina, suprachiasmatic nucleus and intergeniculate leaflet to malnutrition induced during brain development. *Brain Res Bull* 2005;64:395–408.
- Wheaton KL, Hansen KF, Aten S, Sullivan KA, Yoon H, Hoyt KR, et al. The phosphorylation of CREB at serine 133 is a key event for circadian clock timing and entrainment in the suprachiasmatic nucleus. *J Biol Rhythm* 2018;33:497–514.
- Willows R, Sanders MJ, Xiao B, Patel BR, Martin SR, Read J, et al. Phosphorylation of AMPK by upstream kinases is required for activity in mammalian cells. *Biochem J* 2017;474:3059–73.
- Wu R, Dang F, Li P, Wang P, Xu Q, Liu Z, et al. The circadian protein Period2 suppresses mTORC1 activity via recruiting Tsc1 to mTORC1 complex. *Cell Metabol* 2019;29:653–67.
- Yoon MS. mTOR as a key regulator in maintaining skeletal muscle mass. *Front Physiol* 2017;8:788.
- Zhang EE, Liu Y, Dentin R, Pongsawakul PY, Liu AC, Hirota T, et al. Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. *Nat Med* 2010;16:1152.
- Zheng Z, Kim H, Qiu Y, Chen X, Mendez R, Dandekar A, et al. CREBH couples circadian clock with hepatic lipid metabolism. *Diabetes* 2016;65:3369–83.
- Zhong T, Jin PF, Dong EN, Li L, Wang LJ, Zhang HP. Caprine sex affects skeletal muscle profile and MRFs expression during postnatal development. *Anim Sci J* 2013;84:442–8.
- Zhou X, Yan Q, Yang H, Ren A, Kong Z, Tang S, et al. Effects of maternal undernutrition during mid-gestation on the yield, quality and composition of kid meat under an extensive management system. *Animals* 2019a;9:173.
- Zhou X, Yang H, Yan Q, Ren A, Kong Z, Tang S, et al. Evidence for liver energy metabolism programming in offspring subjected to intrauterine undernutrition during midgestation. *Nutr Metab* 2019b;16:20.
- Zhu MJ, Ford SP, Means WJ, Hess BW, Nathanielsz PW, Du M. Maternal nutrient restriction affects properties of skeletal muscle in offspring. *J Physiol* 2006;575:241–50.
- Zhu MJ, Ford SP, Nathanielsz PW, Du M. Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. *Biol Reprod* 2004;71:1968–73.
- Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, et al. AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. *J Physiol* 2008;586:2651–64.