

Intra-uterine undernutrition amplifies age-associated glucose intolerance in pigs via altered DNA methylation at muscle GLUT4 promoter

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Abstract

The present study aimed to investigate the effect of maternal malnutrition on offspring glucose tolerance and the epigenetic mechanisms involved. In total, twelve primiparous Landrace×Yorkshire gilts were fed rations providing either 100% (control (CON)) or 75% (undernutrition (UN)) nutritional requirements according to the National Research Council recommendations, throughout gestation. Muscle samples of offspring were collected at birth (dpn1), weaning (dpn28) and adulthood (dpn189). Compared with CON pigs, UN pigs showed lower serum glucose concentrations at birth, but showed higher serum glucose and insulin concentrations as well as increased area under the blood glucose curve during intravenous glucose tolerance test at dpn189 ($P<0.05$). Compared with CON pigs, *GLUT-4* gene and protein expressions were decreased at dpn1 and dpn189 in the muscle of UN pigs, which was accompanied by increased methylation at the *GLUT4* promoter ($P<0.05$). These alterations in methylation concurred with increased mRNA levels of DNA methyltransferase (*DNMT*) 1 at dpn1 and dpn28, *DNMT3a* at dpn189 and *DNMT3b* at dpn1 in UN pigs compared with CON pigs ($P<0.05$). Interestingly, although the average methylation levels at the muscle *GLUT4* promoter were decreased at dpn189 compared with dpn1 in pigs exposed to a poor maternal diet ($P<0.05$), the methylation differences in individual CpG sites were more pronounced with age. Our results indicate that *in utero* undernutrition persists to silence muscle *GLUT4* likely through DNA methylation during the ageing process, which may lead to the amplification of age-associated glucose intolerance.

Key words: Maternal undernutrition: Ageing: Glucose tolerance: DNA methylation: Glucose transporter-4

Type 2 diabetes and its related risk factors such as glucose intolerance and insulin resistance are an emerging public health problem worldwide⁽¹⁾. Several lines of evidence have shown that undernutrition during early life accelerates the risk of impaired glucose tolerance and type 2 diabetes during adulthood⁽²⁾. In particular, the skeletal muscle has a lower priority for nutrient allocation during fetal development⁽³⁾. When maternal nutrition is poor, the fetuses are adapted to forfeit skeletal muscle development, which is particularly vulnerable to maternal nutrient partitioning for the prioritisation of other very important tissues such as the brain, heart and liver⁽⁴⁾. These early forced adaptations persist later in life, leading to important negative physiological and metabolic consequences in the skeletal muscle, and consequently predispose the offspring to metabolic diseases in adulthood^(5,6).

Deregulation of glucose metabolism is considered as the leading cause for type 2 diabetes mellitus and glucose intolerance⁽⁷⁾. Skeletal muscle predominantly accounts for glucose disposal from the blood, and plays a central role in the

sophisticated regulation of glucose homeostasis⁽⁸⁾. Concretely, glucose uptake in the skeletal muscle is primarily mediated by *GLUT-4*⁽⁹⁾, and glucose is then irreversibly phosphorylated to glucose-6-phosphate by hexokinase (HK)⁽¹⁰⁾. Subsequently, glucose-6-phosphate can be channelled into either the glycolysis pathway catalysed by pyruvate kinase (PK) or the glycogen synthesis pathway catalysed by glycogen synthetase (GS)⁽¹¹⁾. Although the effects of poor nutrition during early life on the expressions of these limited enzymes or transporters in the skeletal muscle of the offspring are well reported in different species^(12–17), all the studies have only examined the acute and short-term effects. It has been shown that because of intra-uterine malnutrition, the fetus is insulin-sensitive at birth and only develops insulin resistance well later in life^(18,19). This suggests that the long-term effects of maternal malnutrition on the onset of metabolic disease in the offspring are highly dependent on the accumulation of additional detrimental events that occur during the ageing process. However, little is known so far about the molecular mechanisms underlying the

Abbreviations: AKT, serine/threonine protein kinase; CON, control; DNMT, DNA methyltransferase; dpn, postnatal day; GS, glycogen synthase; HK2, hexokinase II; IR, insulin receptor; LDH, lactate dehydrogenase; MAT2b, methionine adenosyltransferase 2b; PK, pyruvate kinase; UN, undernutrition.

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interaction between maternal diet and the ageing trajectory in muscle glucose metabolism.

There is increasing evidence that maternal malnutrition can result in permanent changes in fetal gene or protein expressions through epigenetic modulation, thereby leading to increased susceptibility to the related metabolic disease later in life⁽²⁰⁾. In particular, DNA methylation as an important regulatory factor of epigenetics usually occurs in gene-specific promoters and is closely associated with gene activity or silencing⁽²¹⁾. As glucose transport is the first rate-limiting step for glucose storage and utilisation in the skeletal muscle in most instances⁽¹¹⁾, *GLUT4* should be considered as a target gene affected by maternal malnutrition to mediate fetal programming of muscle glucose metabolism. However, there are a limited number of studies demonstrating the effects of maternal nutrition on epigenetic modification of muscle *GLUT4* in the offspring^(13,22). Nevertheless, the existence and the mechanism of the dynamic interaction between these epigenetic changes and age-associated glucose intolerance are unknown.

Compared with rats, pigs are considered better models for nutritional and metabolic studies, because pigs are similar to humans in morphology, physiology, anatomy, metabolism and omnivorous habits^(23,24). It has been reported that pigs with low birth weight exhibit glucose tolerance and insulin resistance during adulthood^(25,26), suggesting that pigs can serve as ideal models to investigate the molecular mechanisms underlying the effects of maternal malnutrition on offspring glucose tolerance. Therefore, the pig was used as a model to answer two questions in this study: (1) to assess the impact of maternal dietary restriction on offspring glucose tolerance and gene expression related to glucose metabolism and insulin signalling in the muscle at different postnatal stages and (2) to assess whether maternal diet modifies age-associated methylation of the *GLUT4* promoter.

Methods

Animal care and experimental design

Animal care and tissue collection were undertaken following the guidelines of the Animal Care and Use Committees at Sichuan Agricultural University. In total, twelve Landrace × Yorkshire gilts (body weight: 148.6 (SEM 6.9) kg) were randomly assigned to the control (CON) and under-nutrition (UN) groups (n 6). The gilts in the CON group were fed 1.0 maintenance requirements of nutrients (M), 1.2 M and 1.5 M diets/d during early gestation (day 1–30 of gestation), middle gestation (day 31–90 of gestation) and late gestation (day 91 of gestation to parturition), respectively, according to the recommendations of the National Research Council (NRC). The gilts in the UN group were provided 75 % of the feed intake of CON gilts at all stages of gestation, respectively. Dietary treatment started after artificial insemination at the fourth oestrus cycle, and the day of the last insemination was defined as day 1 of gestation. All the gilts used in the study were artificially inseminated three times on 2 consecutive days with fresh, pooled semen obtained from two pure Landrace boars, both of which were born of the same sow. After farrowing, the

lactating sows in each group had *ad libitum* access to the same diets until weaning at day 28 of lactation. On postnatal days 1 and 28, six pigs with weights close to the average level (± 10 %) of each litter were selected to be slaughtered. The remaining piglets were adjusted in order to maintain 10–12 pigs/litter in the same group within 24 h after farrowing. The nursing piglets were kept with their mother, offered creep food before weaning and were weaned at 28 d after parturition. At all stages, the gilts were housed individually in feed stalls and had free access to water; they were fed the same diets (online Supplementary Table S1) that were formulated to meet the NRC requirements. After weaning, sixteen (eight male and eight female) weaned piglets originating from six sows in each treatment were selected to be reared until day 189. All the piglets were reared individually following the standard feeding regimen with the starter, grower and finisher diets recommended for the breed. At postnatal day 189, eight pigs that reached mean body weight (SEM 10 %) from each litter were selected to be slaughtered.

Data and sample collection

After farrowing, pigs were weighed on day 1, 28, 68, 110, 152 and 189, and feed intake was recorded every day. On postnatal days 1 (dpn1), 28 (dpn28), 68, 110, 152 and 189 (dpn189), 10 ml of blood was collected from the pigs via the precaval vein in the morning between 07.00 and 08.00 hours before feeding. Serum was harvested by centrifuging the collection tubes at 3000 g for 10 min at 4°C, and the samples were frozen at –80°C until further analysis. The pigs were killed at birth, at weaning and in the adult (dpn189) stage; water was provided *ad libitum*, whereas the feed was removed from the hoppers at 12 h before slaughter. After slaughter, the longissimus dorsi muscle from the left side of the carcass was immediately collected and frozen in liquid N₂.

Intravenous glucose tolerance test

At dpn188, after overnight food deprivation, an intravenous (i.v.) glucose tolerance test (GTT) was conducted according to the method described by Guan *et al.*⁽²⁷⁾. Dextrose (500 g/l) was infused by ear venepuncture at a dose of 0.5 g/kg body weight and an infusion rate of 10 g glucose/min within 6 min. Blood samples were collected at –6, –4, –2 and 0 min relative to completion of dextrose infusion and at 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after infusion. A drop of blood was placed on a test sensor for blood glucose evaluation using a portable Esprit glucometer (Bayer). In addition, glucose clearance rate (k , $\mu\text{mol/l} \times \text{min}$) was calculated by the slope of \log_e glucose against time from 0 to 20 min after an i.v. GTT. The half-life ($T_{1/2}$, min) of glucose was calculated using -0.693 divided by the clearance rate. The area under the blood glucose curve was integrated for each individual pig.

Biochemical parameter analysis

The concentrations of glucose, TAG and cholesterol were determined using commercially available kits (Jiancheng Bioengineering Institute) according to the manufacturer's instructions by an automated biochemical analyser (Model 7020; Hitachi). Serum insulin



concentration was measured using a commercial RIA kit (R&D Systems Europe Ltd).

Metabolites in the muscle

Skeletal muscle tissues were homogenised. The glycogen content of the muscle samples was determined as previously described by Rosenfold *et al.*⁽²⁸⁾, and the results are displayed as milligram of glycogen per gram of muscle (wet weight). Lactate concentration was measured using a commercially available kit (Jiancheng Bioengineering Institute) according to the manufacturer's instructions on a UV-1100 spectrophotometer (Mapada).

Total RNA isolation and RT

Frozen tissue samples (50–100 mg) were crushed in liquid N₂, and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity and quality were determined by agarose gel electrophoresis (1%) and spectrophotometry (A260/A280). RNA concentration was confirmed using the nucleic acid/protein analyser (DU-800; Beckman Coulter Inc.). A commercial RT kit (TaKaRa) was used for complementary DNA (cDNA) synthesis according to the manufacturer's instructions. The RT products (cDNA) were stored at –20°C for relative quantification by PCR.

Real-time quantitative PCR for gene expression analysis

Primers were designed with Primer Express 3.0 (Applied Biosystems) and are shown in the online Supplementary Table S2. cDNA was amplified using the Real-Time PCR System (ABI 7900HT; Applied Biosystems). The reaction mixture (10 µl) contained 5 µl of SYBR Green Supermix (TaKaRa), 1 µl cDNA, 0.4 µl of each primer (10 µM), 0.2 µl ROX Reference Dye and 3 µl of diethylpyrocarbonate-treated water (ddH₂O). The following cycling conditions were used: denaturation at 95°C for 15 s, followed by forty cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. To confirm the specificity of each product, a melting curve analysis (50–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) was performed. All samples were analysed in duplicate; product size was determined by agarose gel electrophoresis. *β-Actin* was used as the internal control for PCR.

Western blot analysis

Total protein was extracted from frozen muscle samples according to the manufacturer's instruction. In brief, samples were homogenised with basic lysis buffer (Beyotime Biotechnology) and protease inhibitor cocktail (Roche). The homogenate samples were then centrifuged for 30 min at 12 000 *g* and 4°C. The supernatant was isolated, and the protein content was measured using a bicinchoninic acid protein assay kit (Beyotime Biotechnology). The protein extract was separated by SDS-PAGE on a 10% gel and transferred to a polyvinylidene fluoride membrane. After the transfer, the membrane was blocked with 5% non-fat dry milk in TBS/T buffer (0.1% Tween, 50 mM-Tris-HCl, 150 mM-NaCl and pH 7.6) for 60 min at room temperature. Later, the membranes were incubated overnight at 4°C with the respective antibodies:

α-tubulin (3873; Cell Signaling) and GLUT4 (ab654; Abcam). After repeated washing with TBS/T buffer, the membranes were incubated with anti-rabbit IgG peroxidase-conjugated secondary antibodies (7074; Cell Signaling) in TBS/T buffer for 60 min at room temperature. Immunoblotted proteins were visualised using the chemiluminescent horseradish peroxidase substrate (Bio-Rad). The relative expression of GLUT4 protein was normalised using *α*-tubulin as the internal protein. The GLUT4 content was presented as the fold change relative to the control group.

DNA extraction

Total DNA from muscle tissues was extracted using a DNAiso reagent kit (TaKaRa), treated with ribonuclease A and purified with the MiniBEST DNA fragment purification kit (TaKaRa). No RNA contamination was detected upon agarose gel electrophoresis, and DNA concentration was measured using a nucleic acid/protein analyser (ND-1000; NanoDrop).

MassARRAY quantitative DNA methylation analysis

The CpG-rich sequences of the GLUT4 promoter regions (online Supplementary Fig. S1) were identified using the University of California Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu/>) (chr12:54810360–54816580, % GC=62% and Obs/Exp CpG=0.85); four EpiTYPER assays were designed using Sequenom EpiDesigner software, which covered 11, 39, 6 and 11 CpG sites, respectively. These assays successfully generated data for 10, 34, 6 and 9 CpG sites, respectively. The primer sequences and the location of the four assays are presented in the online Supplementary Table S3. Quantitative methylation analysis of the porcine GLUT4 promoter was performed using Sequenom's MassARRAY EpiTYPER protocol.

Genomic DNA was treated with bisulphite using the EZ DNA Methylation kit (Zymo Research), according to the manufacturer's instructions. Bisulphite-treated DNA served as a template for amplification, and PCR was performed with the following conditions: 94°C for 4 min followed by forty-five cycles of 94°C for 20 s, 52–62°C for 30 s and 72°C for 3 min. All PCR amplification products were detected on agarose gels before further analysis. After treatment with shrimp alkaline phosphatase, the PCR products were used as templates for *in vitro* transcription and RNase cleavage reaction, as per the manufacturer's instructions. Transcription cleavage products were dispensed into a 384-element SpectroCHIP bioarray, and the mass spectra were acquired by means of a MassARRAY mass spectrometer. The proportion of DNA methylation was calculated by EpiTYPER™ software version 1.0 (Sequenom).

Statistical analysis

Data are presented as mean values with their standard errors. Statistical analysis was performed using SPSS 20.0 software (SPSS Inc.). Serum measurements, gene and protein expressions, as well as methylation levels were analysed as a mixed model with maternal diet and postnatal age as fixed effects using the MIXED procedure in SPSS. During the GTT, both diet and time point were considered as the fixed effects. One-way ANOVA, followed by Duncan's multiple-range test, was

conducted to determine the differences between treatments. In addition, sow reproductive performance and piglet growth performance were analysed by independent-samples *t* test. Relative mRNA abundance was determined using the $\Delta\Delta C_t$ threshold ($\Delta\Delta C_t$) method, and results of the relative mRNA abundance are expressed as fold change relative to the mean value of the control group. Probability values <0.05 were considered as statistically significant.

Results

Growth performance

The mean birth weight and mean litter weight were lower in the UN group than in the CON group (Table 1, $P<0.01$). Although lower body weights were observed in UN pigs than in CON pigs at dpn28 ($P<0.05$), body weights were not affected at dpn68, dpn110, dpn152 and dpn189 among treatments (Table 2). There was no difference in average food intake during 28–68, 68–110, 110–152, 152–189 and 29–189 d between the UN and CON groups (Table 3).

Serum metabolites, serum hormones and muscle metabolites

Serum glucose concentration was significantly lower in UN pigs at dpn1 (Table 4, $P<0.05$), but higher ($P<0.05$) at dpn152 and dpn189 than in CON pigs. Compared with CON pigs, serum TAG concentration was significantly increased ($P<0.05$) in UN pigs at dpn152. Elevation in serum insulin concentrations was observed in UN pigs at dpn110 ($P<0.05$) and dpn189 ($P<0.05$), compared with that in CON pigs. In addition, UN pigs exhibited higher muscle lactate content at dpn1 ($P<0.05$), and lower muscle glycogen content at dpn28 ($P<0.05$) and dpn189 ($P<0.05$), compared with their counterparts in the CON group. In UN pigs, serum glucose content was lower at day 1 than at days 28, 68, 110, 152 and 189, whereas in CON pigs serum glucose content was lower at day 1 than at days 28, 68 and 110 ($P<0.05$). Compared with day 1, serum TAG and cholesterol contents increased at day 28 in both UN and CON pigs ($P<0.05$). High serum insulin content was observed at day 189 rather than at days 1, 28, 68, 110 and 152 in UN pigs,

whereas high serum insulin content was observed at day 1 rather than at days 68, 110 and 152 in CON pigs ($P<0.05$). Compared with day 1, glycogen content was decreased and lactate content was increased in the muscle of both UN and CON pigs at day 189 ($P<0.05$).

Intravenous glucose tolerance test

Blood glucose concentration was significantly higher in UN pigs at post-infusion times 5 and 20 than that in CON pigs (online Supplementary Table S4, $P<0.05$). During i.v.GTT, the area

Table 2. Body weight of pigs at different stages (Mean values with their standard errors; *n* 16 pigs per treatment)

	CON group		UN group	
	Mean	SEM	Mean	SEM
Body weight (kg)				
Day 1	1.38	0.02	1.03*	0.03
Day 28	6.68	0.10	6.34*	0.12
Day 68	21.38	0.60	20.71	0.61
Day 89	38.82	0.76	38.35	1.23
Day 110	54.93	1.27	56.70	2.22
Day 131	71.63	2.01	71.01	2.16
Day 152	89.83	2.81	90.24	2.37
Day 189	123.33	2.72	121.01	4.03

CON, control; UN, undernutrition.

* Significant differences between control and undernutrition groups ($P<0.05$).

Table 3. Growth performance of pigs at different stages (Mean values with their standard errors; *n* 16 pigs per treatment)

	CON group		UN group	
	Mean	SEM	Mean	SEM
Average daily gain (g)				
Day 28–68	367	16	358	16
Day 68–89	831	41	840	34
Day 89–110	767	50	874	50
Day 110–131	796	46	682	29
Day 131–152	866	42	916	50
Day 152–189	906	8	832	59
Day 28–189	724	16	712	25
Average daily feed intake (g)				
Day 28–68	703	30	642	33
Day 68–89	1526	64	1612	63
Day 89–110	1902	102	2014	116
Day 110–131	2280	79	2186	92
Day 131–152	2746	150	3242	215
Day 152–189	2799	123	2883	64
Day 28–189	1921	52	2003	60
Feed conversion ratio†				
Day 28–68	0.523	0.010	0.561	0.015
Day 68–89	0.546	0.023	0.522	0.013
Day 89–110	0.403	0.017	0.434	0.010
Day 110–131	0.349	0.016	0.313	0.013
Day 131–152	0.318	0.015	0.286	0.016
Day 152–189	0.327	0.016	0.287	0.016
Day 28–189	0.378	0.006	0.356*	0.007

CON, control; UN, undernutrition.

* Significant differences between control and undernutrition groups ($P<0.05$).

† Feed conversion ratio calculated by the ratio of average daily gain:average daily feed intake.

Table 1. Sow performance and body weight of killed piglets (Mean values with their standard errors; *n* 6 litter per treatment)

	CON group		UN group	
	Mean	SEM	Mean	SEM
Litter size (<i>n</i>)	14.50	0.43	14.00	0.52
Litter weight (kg)	19.50	0.47	15.20*	0.55
Birth weight (kg)	1.35	0.03	1.09*	0.03
Birth weight for killed piglets†				
Piglets killed at birth (kg)	1.34	0.02	1.05*	0.03
Piglets killed at weaning (kg)	1.39	0.03	1.01*	0.02
Piglets killed in adult (kg)	1.38	0.02	1.06*	0.04

CON, control; UN, undernutrition.

* Significant differences between control and undernutrition groups ($P<0.05$).

† Piglets killed at birth (postnatal day 1), weaning (postnatal day 28) and adulthood (postnatal day 189).

Table 4. Serum and muscle metabolites of pigs at different stages (Mean values with their standard errors; *n* 16 pigs per treatment)

	CON group		UN group		<i>P</i>		
	Mean	SEM	Mean	SEM	Diet	Time	Diet × time
Serum glucose (mmol/l)							
Day 1	4.56 ^{a,D}	0.23	2.87 ^{b,B}	0.47	0.63	<0.01	0.04
Day 28	8.03 ^A	0.42	6.94 ^A	0.78			
Day 68	5.99 ^{B,C}	0.38	6.87 ^A	0.78			
Day 110	6.84 ^{A,B}	0.59	6.98 ^A	0.62			
Day 152	5.13 ^{b,C,D}	0.30	6.68 ^{a,A}	0.49			
Day 189	4.71 ^{b,C,D}	0.39	5.85 ^{a,A}	0.21			
Serum TAG (mmol/l)							
Day 1	0.42 ^C	0.10	0.54 ^C	0.11	0.06	<0.01	0.42
Day 28	0.85 ^{A,B}	0.07	1.00 ^{A,B}	0.10			
Day 68	0.53 ^{B,C}	0.06	0.53 ^C	0.03			
Day 110	0.63 ^{A,B,C}	0.12	0.75 ^{B,C}	0.17			
Day 152	0.88 ^{b,A}	0.11	1.29 ^{a,A}	0.14			
Day 189	0.52 ^{B,C}	0.05	0.51 ^C	0.05			
Serum cholesterol (mmol/l)							
Day 1	1.27 ^C	0.13	1.12 ^C	0.14	0.44	<0.01	0.63
Day 28	4.34 ^A	0.62	5.01 ^A	0.51			
Day 68	2.23 ^B	0.06	2.26 ^B	0.12			
Day 110	2.42 ^B	0.04	2.54 ^B	0.21			
Day 152	2.17 ^B	0.02	2.23 ^B	0.09			
Day 189	2.34 ^B	0.12	2.25 ^B	0.04			
Serum insulin (mmol/l)							
Day 1	26.79 ^A	1.08	26.69 ^B	1.31	0.02	<0.01	0.03
Day 28	21.24 ^{A,B}	0.84	22.81 ^B	1.06			
Day 68	17.01 ^{B,C}	2.22	17.76 ^B	1.92			
Day 110	12.89 ^{b,C}	0.97	18.76 ^{a,B}	2.36			
Day 152	18.37 ^{B,C}	2.13	17.57 ^B	4.21			
Day 189	22.05 ^{b,A,B}	3.85	36.25 ^{a,A}	4.58			
Muscle glycogen (mg/g tissue)							
Day 1	63.72 ^A	4.62	56.54 ^A	4.22	0.15	<0.01	0.64
Day 28	7.33 ^{a,B}	0.60	4.88 ^{b,B}	0.18			
Day 189	6.27 ^{a,B}	0.26	4.30 ^{b,B}	0.26			
Muscle lactate (mmol/g protein)							
Day 1	1.16 ^{b,B}	0.06	1.56 ^{a,B}	0.19	0.73	<0.01	0.50
Day 28	2.44 ^A	0.23	2.50 ^A	0.41			
Day 189	2.36 ^A	0.09	2.48 ^A	0.10			

CON, control; UN, undernutrition.

^{a,b} Mean values within a row with unlike superscript lower-case letters were significantly different between diet groups ($P < 0.05$).

^{A,B,C,D} Mean values within parameter and diet with unlike superscript capital letters were significantly different between times ($P < 0.05$).

under the blood glucose curve (AUC, $P < 0.05$) and half-life ($P < 0.05$) were significantly increased, whereas glucose clearance rate ($P < 0.05$) was significantly decreased in UN pigs, compared with those in CON pigs (Fig. 1).

Gene expression involved in muscle glucose metabolism

At dpn1, the expressions of *GLUT4* and *HK2* mRNA were significantly down-regulated (Table 5, $P < 0.05$), whereas the expression of lactate dehydrogenase (*LDH*) mRNA was significantly up-regulated in the skeletal muscle of UN pigs, as compared with those in CON pigs. At dpn28, maternal undernutrition during gestation markedly ($P < 0.05$) decreased mRNA levels of *GLUT4*, *GS*, *PK* and citrate synthase (*CS*) and increased *LDH* mRNA levels in the offspring skeletal muscle. At dpn189, the expression levels of *GS* and *GLUT4* were significantly lower ($P < 0.05$), whereas the expression level of pyruvate dehydrogenase kinase (*PDK*) was significantly ($P < 0.05$) higher in the UN group than in the CON group. In UN

pigs, *PK* mRNA levels were lower at day 1 than at day 28, and *LDH* mRNA levels were higher at day 28 than at day 189 ($P < 0.05$). In CON pigs, *GLUT4* mRNA level was higher but *GS* mRNA level was lower at day 1 than at days 28 and 189, whereas *PK*, *CS* and *PDK* mRNA levels were lower at day 1 than at day 28 ($P < 0.05$).

Gene expression involved in muscle insulin signalling

At dpn1, the expressions of insulin receptor (*IR*) and serine/threonine protein kinase (AKT) 2 (*AKT2*) mRNA were significantly down-regulated (Table 6, $P < 0.05$). At dpn28, the expression levels of insulin receptor substrate 1 (*IRS1*) and phosphatidylinositol 3-kinase (*PI3K*) were significantly lower (Table 6, $P < 0.05$) in the UN group than in the CON group. At dpn189, the *IR* transcript level was significantly decreased ($P < 0.05$) in the skeletal muscle of UN pigs. In UN pigs, *IR* mRNA level was higher at day 28 than at days 1 and 189, whereas *AKT2* mRNA level was lower at day 1 than at days 28

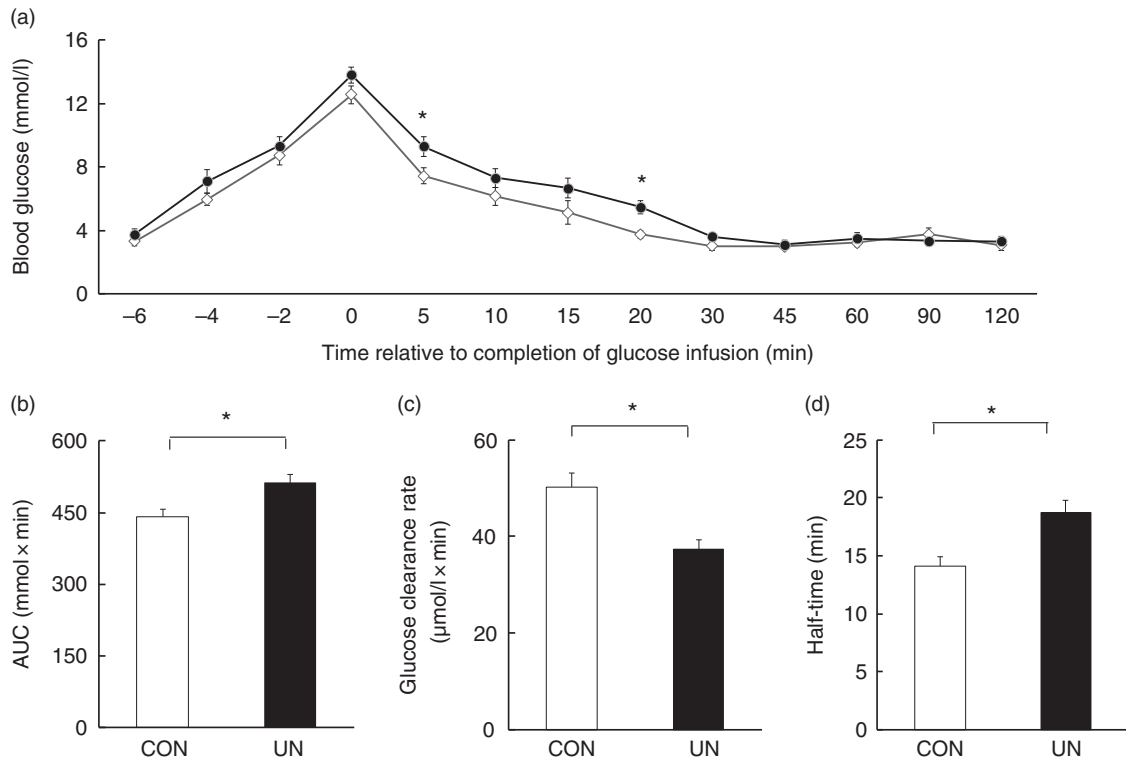


Fig. 1. Plasma glucose concentrations (a), area under the blood glucose curve (AUC, b), glucose clearance rate (c) and half-time (d) during intravenous glucose tolerance test in pigs from control (CON) or undernutrition (UN) dams. Time indicates minutes relative to completion of dextrose infusion (n 8). * Significantly different between UN and CON group ($P < 0.05$). —◇—, CON; —●—, UN.

Table 5. The mRNA expressions of genes related to glucose metabolism in skeletal muscle of pigs at different stages (Mean values with their standard errors; n 6 at days 1 and 28, n 8 at day 189)

	Time	CON group		UN group		<i>P</i>		
		Mean	SEM	Mean	SEM	Diet	Time	Diet x time
<i>GLUT4</i>	Day 1	1.00 ^{a,A}	0.13	0.26 ^b	0.03	<0.01	<0.01	<0.01
	Day 28	0.40 ^{a,B}	0.05	0.18 ^b	0.02			
	Day 189	0.62 ^{a,B}	0.07	0.26 ^b	0.04			
<i>HK2</i>	Day 1	1.00 ^a	0.14	0.39 ^b	0.10	0.05	0.05	0.92
	Day 28	2.16	0.60	1.31	0.36			
	Day 189	1.60	0.50	1.03	0.15			
<i>GS</i>	Day 1	1.00 ^B	0.11	1.18	0.13	0.04	<0.01	0.01
	Day 28	2.17 ^{a,A}	0.42	1.01 ^b	0.12			
	Day 189	2.23 ^{a,A}	0.35	1.13 ^b	0.14			
<i>PK</i>	Day 1	1.00 ^B	0.12	0.56 ^B	0.22	<0.01	0.03	0.08
	Day 28	4.05 ^{a,A}	0.83	1.82 ^{b,A}	0.52			
	Day 189	1.44 ^B	0.47	1.32	0.10			
<i>LDH</i>	Day 1	1.00 ^b	0.09	3.06 ^a	1.00	0.03	<0.01	0.14
	Day 28	1.42 ^b	0.32	3.48 ^{a,A}	0.22			
	Day 189	0.97	0.29	1.29 ^B	0.24			
<i>CS</i>	Day 1	1.00 ^B	0.11	1.20	0.31	0.08	0.32	0.09
	Day 28	2.44 ^{a,A}	0.36	0.98 ^b	0.29			
	Day 189	1.68	0.55	1.83	0.46			
<i>PDK</i>	Day 1	1.00 ^B	0.16	1.89	0.40	0.15	0.29	0.04
	Day 28	2.63 ^A	0.52	1.72	0.50			
	Day 189	0.93 ^{b,B}	0.25	2.05 ^a	0.36			

CON, control; UN, undernutrition; *HK2*, hexokinase II; *GS*, glycogen synthase; *PK*, pyruvate kinase; *LDH*, lactate dehydrogenase; *CS*, citrate synthase; *PDK*, pyruvate dehydrogenase kinase.

^{a,b} Mean values within a row with unlike superscript lower-case letters were significantly different between diets ($P < 0.05$).

^{A,B} Mean values within parameter and diet with unlike superscript capital letters were significantly different between times ($P < 0.05$).

Table 6. The mRNA expressions of genes related to insulin signalling in skeletal muscle of pigs at different stages (Mean values with their standard errors; *n* 6 at days 1 and 28, *n* 8 at day 189)

	Time	CON group		UN group		<i>P</i>		
		Mean	SEM	Mean	SEM	Diet	Time	Diet × time
<i>IR</i>	Day 1	1.00 ^a	0.09	0.67 ^{b,B}	0.08	0.05	<0.01	0.04
	Day 28	1.28	0.21	1.17 ^A	0.18			
	Day 189	1.59 ^a	0.29	0.59 ^{b,B}	0.06			
<i>IRS1</i>	Day 1	1.00 ^B	0.18	0.80	0.16	0.07	<0.01	0.03
	Day 28	1.86 ^{a,A}	0.25	0.72 ^b	0.07			
	Day 189	1.24	0.19	0.72	0.13			
<i>PI3K</i>	Day 1	1.00	0.24	1.45	0.59	0.30	0.73	0.76
	Day 28	0.89 ^a	0.12	0.39 ^b	0.05			
	Day 189	0.88	0.17	0.68	0.12			
<i>AKT1</i>	Day 1	1.00	0.30	1.07	0.32	0.44	0.10	0.78
	Day 28	0.90	0.25	0.62	0.15			
	Day 189	1.17	0.20	1.16	0.10			
<i>AKT2</i>	Day 1	1.00 ^a	0.11	0.59 ^{b,B}	0.06	0.15	0.75	0.29
	Day 28	1.15	0.31	0.89 ^A	0.11			
	Day 189	1.04	0.23	0.91 ^A	0.07			
<i>GSK3β</i>	Day 1	1.00	0.19	1.73	0.44	0.63	0.05	0.66
	Day 28	1.37 ^A	0.20	1.62	0.26			
	Day 189	0.55 ^B	0.15	1.44	0.62			

CON, control; UN, undernutrition; *IR*, insulin receptor; *IRS1*, insulin receptor substrate 1; *PI3K*, phosphatidylinositol 3-kinase; *AKT1/2*, serine/threonine protein kinases 1/2; *GSK3*, glycogen synthase kinase 3.

^{a,b} Mean values within a row with unlike superscript lower-case letters were significantly different between diets ($P < 0.05$).

^{A,B} Mean values within parameter and diet with unlike superscript capital letters were significantly different between times ($P < 0.05$).

Table 7. The mRNA expressions of genes related to DNA methylation in skeletal muscle of pigs at different stages (Mean values with their standard errors; *n* 6 at days 1 and 28, *n* 8 at day 189)

	Time	CON group		UN group		<i>P</i>		
		Mean	SEM	Mean	SEM	Diet	Time	Diet × time
<i>DNMT1</i>	Day 1	1.00 ^b	0.26	3.15 ^{a,A}	0.43	0.03	<0.01	0.10
	Day 28	1.45 ^b	0.22	2.70 ^a	0.44			
	Day 189	0.88	0.22	1.35 ^B	0.49			
<i>DNMT3a</i>	Day 1	1.00	0.49	0.73	0.25	0.68	0.35	0.29
	Day 28	0.48 ^b	0.11	0.94 ^a	0.18			
	Day 189	0.42 ^b	0.08	0.85 ^a	0.06			
<i>DNMT3b</i>	Day 1	1.00	0.19	2.33	0.28	0.09	0.13	0.39
	Day 28	2.15	0.54	2.33	0.69			
	Day 189	0.97	0.23	1.27	0.67			
<i>BHMT</i>	Day 1	1.00	0.36	0.89 ^B	0.08	0.05	0.08	0.03
	Day 28	0.57	0.20	0.59 ^B	0.18			
	Day 189	0.60 ^b	0.06	1.64 ^{a,A}	0.20			
<i>MAT2b</i>	Day 1	1.00	0.20	1.06 ^B	0.09	0.01	0.33	0.41
	Day 28	2.08	0.84	3.54 ^A	1.09			
	Day 189	0.99	0.28	0.99 ^B	0.38			
<i>AHCY1</i>	Day 1	1.00 ^A	0.20	0.86	0.09	0.03	0.59	0.20
	Day 28	0.63	0.84	1.09 ^A	1.09			
	Day 189	0.46 ^B	0.28	0.39 ^B	0.38			

CON, control; UN, undernutrition; *DNMT1/3a/3b*, DNA methyltransferase 1/3a/3b; *BHMT*, betaine-homocysteine methyltransferase; *MAT2b*, methionine adenosyltransferase 2b; *AHCY1*, adenosylhomocysteine 1.

^{a,b} Mean values within a row with unlike superscript lower-case letters were significantly different between diets ($P < 0.05$).

^{A,B} Mean values within parameter and diet with unlike superscript capital letters were significantly different between times ($P < 0.05$).

and 189 ($P < 0.05$). In CON pigs, *IRS1* mRNA level was lower at day 1 than at day 28, whereas glycogen synthase kinase 3β (*GSK3β*) mRNA level was higher at day 28 than at day 189 ($P < 0.05$).

Gene expression involved in DNA methylation

At dpn1, DNA methyltransferase (*DNMT*) 1 and *DNMT3b* were expressed at higher (Table 7, $P < 0.05$) levels in the UN group compared with the CON group. At dpn28, the transcript levels

of *DNMT1* and *DNMT3a* were significantly increased ($P < 0.05$) in UN pigs compared with CON pigs. At dpn189, the mRNA levels of *DNMT3a* and betaine-homocysteine methyltransferase (*BHMT*) were significantly higher ($P < 0.05$) in UN pigs than in CON pigs. In UN pigs, *DNMT1* mRNA level was higher at day 1 than at day 189, and adenosylhomocysteine 1 (*AHCY1*) mRNA level was higher at day 28 than at day 189 ($P < 0.05$). In CON pigs, *AHCY1* mRNA level was higher at day 1 than at day 189 ($P < 0.05$).

Protein content of muscle GLUT4

Compared with CON pigs, the protein content of GLUT4 (Fig. 2) was decreased in the skeletal muscle of UN pigs at dpn1 and dpn189 ($P < 0.05$).

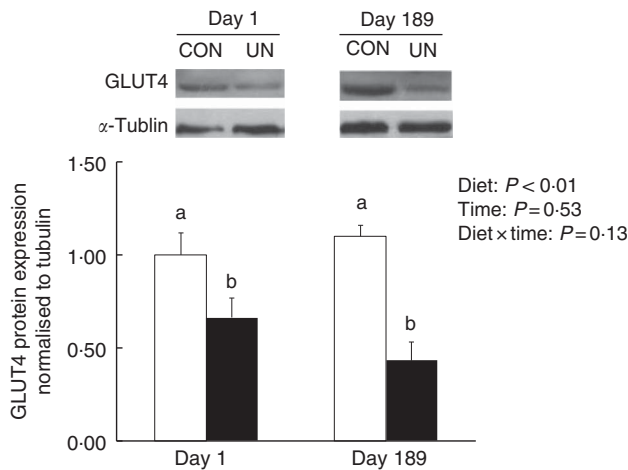


Fig. 2. Protein expression of GLUT-4 in skeletal muscle of pigs from control (CON) or undernutrition (UN) dams at postnatal days 1 and 189. Values are means (n 4 per treatment), with standard errors represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). □, CON; ■, UN.

DNA methylation at the GLUT4 promoter region

At dpn1, the sequencing results of the *GLUT4* promoter indicated that the methylation ratio was significantly increased at -128, -661 and -826 sites in the UN pigs (Fig. 3, $P < 0.05$) compared with CON pigs. At dpn189, the methylation levels at -128, -826, -840 and -909 sites of the sequenced *GLUT4* promoter showed a significant rise in UN pigs compared with the CON pigs ($P < 0.05$). In addition, the average methylation level was significantly increased in UN pigs at dpn1 ($P < 0.05$) and dpn189 ($P < 0.05$) compared with CON pigs. In UN pigs, the average methylation level was higher at day 1 than at day 189 ($P < 0.05$).

Discussion

In agreement with previous reports in rodents⁽²⁹⁾, ewes⁽³⁰⁾ and humans⁽³¹⁾, this study provided the first evidence in a pig model that maternal undernutrition during pregnancy indeed induced moderate diabetes mellitus and impaired glucose intolerance during adulthood, as demonstrated by increased serum glucose level, insulin content and AUC glucose in adult UN pigs. These outcomes are in agreement with the notion that maternal malnutrition exerts permanent effects on offspring metabolic

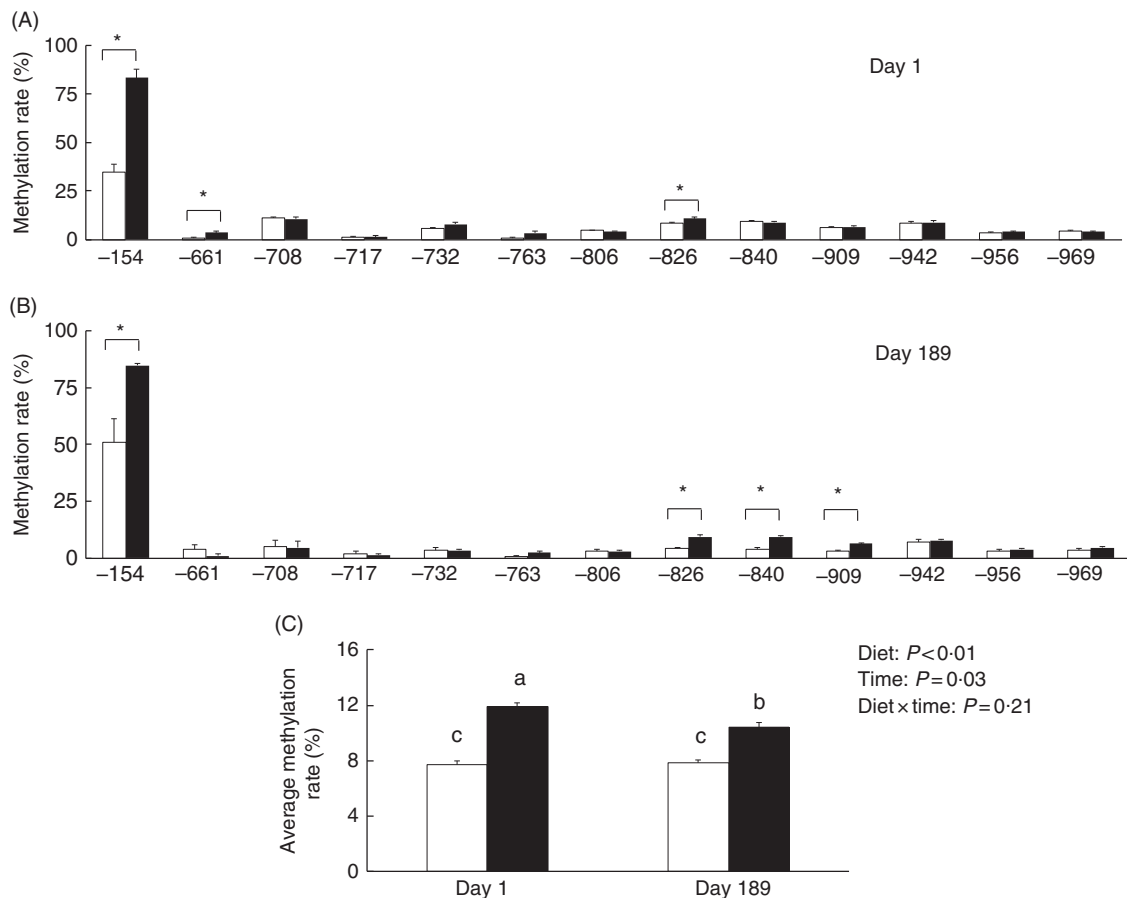


Fig. 3. Methylation of individual CpG sites at the *GLUT-4* promoter region in the skeletal muscle of pigs from control (CON) or undernutrition (UN) dams at postnatal day 1 (A) and day 189 (B). (C) Compare the percentage of mean methylation at 1–13 CpG sites between UN and CON groups. Values are means (n 4 per treatment), with standard errors represented by vertical bars. * Significantly different between treatments ($P < 0.05$). ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). □, CON; ■, UN.

diseases later in life⁽²⁾. Interestingly, studies in rats⁽³²⁾, sheep^(33,34) and humans^(35,36) have shown that intra-uterine malnutrition or low birth weight offspring displayed improved insulin sensitivity very early during postnatal life. However, a shift towards decreased insulin sensitivity and impaired glucose tolerance is evident in later life as these offspring age^(18,19). Consistently, we found that pigs from malnourished dams had lower serum glucose content at birth, but developed hyperglycaemia and hyperinsulinaemia as adults. This opposite shift suggested a significant interaction between maternal diet and ageing effects.

In particular, skeletal muscles, which account for 70–80% of postprandial glucose disposal, play a central role in the maintenance of whole-body glucose homeostasis⁽⁸⁾. Impaired glucose intolerance is strongly associated with deregulation of glucose disposal in the skeletal muscle⁽⁸⁾. GLUT4 accounts for the rate-limiting step in glucose uptake, and is the port of entry for glucose into the muscle. A defect in muscle GLUT4 causes severe insulin resistance and impaired glucose intolerance^(37,38). Interestingly, we found that muscle *GLUT4* mRNA and protein expressions in UN pigs were persistently decreased from birth to adulthood. This suggests that maternal malnutrition displays a long-lasting impact on muscle *GLUT4* expression, thereby contributing to the development of glucose intolerance in adults.

However, the effects of maternal malnutrition on GLUT4 varied in different previous studies. In early life, a food-deprived (50 *v.* 100%) diet during pregnancy and lactation resulted in decreased GLUT4 protein content in the muscle of rats at postnatal days 2 and 60⁽¹⁵⁾. Consistently, in sheep, placental restriction decreased protein content of muscle GLUT4 at 140 d of gestation⁽¹⁶⁾ or decreased the mRNA level of muscle *GLUT4* at 43 (SEM 2) d of age⁽¹⁴⁾. Conversely, a similarly designed experiment demonstrated a contrasting result that showed increased muscle GLUT4 protein content in 38-d-old female rats derived from dams fed a low-protein (LP) diet (8 *v.* 20%) during gestation and lactation⁽¹³⁾, or in placentally restricted lamb at 21 d of age⁽¹⁶⁾. Results from subcellular fractionation experiments were also inconsistent. Non-stimulated or insulin-stimulated glucose uptake and GLUT4 translocation in the muscle were reported to increase in rats at 60⁽¹⁷⁾ and 90⁽³⁹⁾ d, or decrease in rats at 70 d⁽⁴⁰⁾, which were derived from dams fed LP or food-restricted diet. In particular, Freitas *et al.*⁽⁴¹⁾ reported that insulin-stimulated muscle GLUT4 translocation was decreased at 4 and 8 d, but was increased at 60 d in rats from dams that received a protein-free diet during lactation.

In later postnatal life, the effects of maternal malnutrition on GLUT4 displayed consistency across different species. In rat studies, GLUT4 protein content was decreased in the muscle of 15-month-old rats, which were born from LP dams⁽⁴¹⁾ or placentally restricted dams⁽⁴²⁾. Consistently, in human studies, reduced GLUT4 protein content was observed in young men with low birth weight^(43,44). Generally, early postnatal life is characterised by immaturity and plasticity, which then progresses to altered glucose homeostasis from growth to maturity in adults. Therefore, diverse GLUT4 changes in different species during early life induced by poor prenatal nutrition reflect short-term and diverse adaptive responses to

improved environmental cues after birth, acting through developmental plasticity. However, the long-term effects of GLUT4 attributed by nutritional factors acting *in utero* can be fixed in adulthood possibly through epigenetic modulations, which in turn confer an adverse phenotype.

Emerging evidences have revealed that intra-uterine under-nutrition leads to permanent changes in the expressions of genes or proteins via DNA methylation, which can alter susceptibility to complex diseases later in life⁽⁴⁵⁾. In this study, analysis of DNA methylation patterns in the promoter region of *GLUT4* indicated a significant increase in methylation levels in newborn and adult UN pigs. Typically, hypermethylation at the gene promoter is considered to prevent gene expression^(46,47). Therefore, these observations of hypermethylation at the *GLUT4* promoter are in good agreement with the trend of progressive decline in muscle *GLUT4* transcription in newborn and adult UN pigs. Therefore, our findings suggest that altered methylation at muscle GLUT4 promoter persists throughout the lifetime of the pigs, even without further exposure to poor nutrition after birth. It is considered that DNA methylation displays long-term impacts on transcriptional regulation of muscle *GLUT4*, consequently contributing to impaired glucose intolerance during adulthood.

Indeed, it has been shown that total methylcytosine content is prone to decrease upon ageing, which underlies reduced genomic hypomethylation^(48,49). This suggests that ageing is critical for the regulation of DNA methylation. A body of literature has demonstrated the changes in epigenetic patterns over the ageing process. Park *et al.*⁽⁵⁰⁾ reported that histone acetylation was progressively lost, whereas histone methylation (H3K9me2) was progressively strengthened, following intra-uterine growth retardation during the ageing process. Consistently, an interesting study by Sandovici *et al.*⁽⁵¹⁾ showed that ageing leads to progressively suppressive histone marks of the hepatocyte nuclear factor-4 in the islets, and a poor maternal diet amplifies the age-associated epigenetic silencing of this locus. A similar observation was also reported by Murgatroyd & Avwu⁽⁵²⁾. These studies indicate that early poor nutrition can interact with ageing to influence gene and protein expressions, thereby leading to the development of adverse metabolic phenotypes in later life. Interestingly, in this study, the average methylation levels at the *GLUT4* promoter were lower in adults than at birth in UN pigs, but not in CON pigs. Meanwhile, the differences in CpG-site methylation were more pronounced in adults (at –128, –826, –840 and –909) than at birth (at –128, –661 and –826). Both these observations suggested that the methylation changes occurring with age were highly dependent on maternal nutrition status.

Indeed, DNMT, an enzyme that catalyses the transfer of a methyl group to DNA, is required for establishing genomic methylation patterns⁽⁵³⁾. In this study, *DNMT1* expression persistently increased in the skeletal muscle of both newborn and weaning UN pigs, whereas *DNMT3b* and *DNMT3a* increased in the skeletal muscle of newborn and adult UN pigs, respectively. Reduced expression of *DNMT* in the skeletal muscle of UN pigs might be expected to support hypermethylation at the *GLUT4* promoter during postnatal life. In addition, it has been proposed that decreased *DNMT1* expression

underlies reduced genomic hypomethylation during the ageing process. As expected, we found that UN pigs showed decreased muscle *DNMT1* expression as the postnatal age progressed. These changes may help UN pigs to reduce methylation levels at the *GLUT4* promoter with age. In addition, BHMT, which is an enzyme limited to methyl-group metabolism, was increased in the skeletal muscle of UN pigs at dpn189, but not at dpn1 and 28. We speculate that UN offspring might be prenatally adapted to a nutrient-deficient environment, and therefore may respond postnatally to an adequate nutrient supply with increased catabolism of the methyl group by BHMT. This alteration in *BHMT* expression may help UN pigs to maintain high methylation levels in later life.

Once glucose is transported into the skeletal muscle by GLUT4, it can be phosphorylated to glucose-6-phosphate catalysed by HK2. It has been shown that decreased muscle glucose uptake is associated with decreased activity or expression of HK2⁽⁵⁴⁾, indicating that muscle glucose transport may be rate limiting for HK2 expression. As expected, we found that muscle *HK2* expression was down-regulated in UN pigs at dpn1. Subsequently, most of the glucose-6-phosphate should be stored as muscle glycogen, which is mainly catalysed by GS, an enzyme that is rate limiting for glycogen synthesis⁽⁵⁵⁾. It has been shown that impaired glycogen synthesis is the key feature of muscle insulin resistance in patients with type 2 diabetes mellitus⁽⁵⁶⁾. Consistently, our present study showed that impaired glucose tolerance was associated with decreased muscle glycogen synthesis in UN pigs, as demonstrated by the reduction in muscle *GS* expression and glycogen content at dpn28 and dpn189.

Beside glycogen synthesis, a fraction of glucose-6-phosphate is further processed in glycolysis⁽¹¹⁾. In particular, PK is a key glycolytic enzyme that catalyses the final step of glycolysis with the generation of pyruvate⁽⁵⁷⁾. Further, pyruvate can be catalysed to coenzyme A, which is the metabolic substrate for the tricarboxylic acid cycle (TCA)⁽⁵⁸⁾. Consequently, the altered PK expression would be expected to influence glucose oxidation and energy production. Our present study provides the first evidence that muscle *PK* mRNA level was reduced in UN pigs at dpn28, indicating that maternal undernutrition may impair offspring glucose oxidation in the skeletal muscle. As expected, CS, an enzyme that is the first rate limiting for TCA⁽⁵⁹⁾, was reduced in UN pigs at dpn28, and PDK4, an enzyme that participates in the suppression of glucose oxidation through inhibition of pyruvate dehydrogenase⁽⁶⁰⁾, was observed to be increased in UN pigs at dpn189. In addition, in glycolytic muscles with a low oxidative capacity, most of the pyruvate can be catalysed to lactate by LDH with the generation of ATP⁽¹¹⁾. Our results showed that the mRNA level of muscle *LDH* was increased in UN pigs at dpn1 and dpn28; this indicated an adaptation to help UN offspring stimulate ATP production to promote muscle growth. We also found that increased *LDH* expression is associated with increased lactate content in the skeletal muscle of UN pig at dpn1. This alteration would be expected to weaken muscle insulin sensitivity, as increased lactate leads to induction of muscle insulin resistance⁽⁶¹⁾. Besides the effects of maternal diet, *PK* expression was up-regulated at day 28 compared with day 1, suggesting a

higher nutrient supply at day 28 than at day 1, as PK is sensitive to nutrient supply⁽⁶²⁾. Therefore, we assume that the differences in *PK* expression were obvious at day 28, as the UN fetuses were not adapted to cope with improved nutritional environment after birth. In addition, *LDH* expression in UN pigs was lower at day 189 than at days 1 and 28. This suggested a deficiency of energy at days 1 and 28, as LDH stimulates ATP production⁽¹¹⁾. Therefore, we assume that the differences in *LDH* expression were significant at days 1 and 28 because the fetuses in the UN group were not able to meet their energy requirements completely. However, as these offspring grew with an improved energy supply, changes in *LDH* expression were not obvious during adulthood.

Notably, insulin signalling plays a major role in controlling glucose homeostasis in almost all the body tissues, and impaired insulin signalling is associated with insulin resistance⁽⁶³⁾. The major signal proteins in this cascade include IR, IRS1/IRS2, PI3K and AKT1/AKT2⁽⁷⁾. Impaired insulin signalling can result in two insulin responses in the skeletal muscle: decreased glycogen synthesis via phosphorylation of GSK3 β or limited glucose transfer via translocation of GLUT4⁽⁶³⁾. Therefore, in this study, UN pigs exhibited decreased muscle insulin sensitivity as confirmed by decreased expressions of *IR*, *IRS1*, *PI3K* and *AKT1* at different postnatal ages. These changes would be expected to impair GLUT4 and GS expressions. However, the effects of maternal malnutrition on insulin signalling vary in different stages. During fetal life, *IR* expression was improved in the skeletal muscle of sheep subjected to placental insufficiency^(16,64). In early postnatal age, the responses to insulin signalling are shown to differ in previous studies, with reports of decrease at 42 d in sheep from placentally restricted dams⁽¹⁴⁾, increase at 21 d in sheep from placentally restricted dams⁽¹⁶⁾ and at 60 d in rats from under-nourished dams^(17,41), or unchanged at 60 d in rats from under-nourished dams⁽⁶⁵⁾. As insulin signalling is strongly related to the insulin content, up-regulation of insulin signalling is considered as a natural compensatory response to low insulin levels in all insulin-responsive cells and organisms with an under-nourished fetus. Therefore, the inconsistent response of insulin signalling in our study may be due to the unchanged insulin content at birth. In later life, low birth weight subjects showed reduced expressions of several proteins related to insulin signalling in the muscle^(43,44), which is in agreement with our result. A similar response was observed in rats as well⁽⁴³⁾. These changes help in the development of age-associated glucose intolerance during adulthood.

Conclusion

In conclusion, our results indicate that maternal undernutrition during pregnancy decreases *GLUT4* gene and protein expressions in the muscle of offspring likely by favouring methylation of the muscle *GLUT4* promoter. Further, these alterations in methylation can persist into adulthood with long-term consequences on glucose homeostasis and insulin sensitivity in the skeletal muscle, possibly contributing to subsequent pathogenesis of glucose intolerance in adult life. In addition, ageing can progressively interact with maternal diet to influence methylation of the muscle *GLUT4* promoter, which may help in

the amplification of age-associated glucose intolerance induced by the maternal diet.

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The authors' contributions are as follows: D. W. and J. W. designed the study; J. W. wrote the paper; J. W., M. C., M. Y. and J. L. carried out the animal study; J. W. and Y. L. performed the analysis of biochemical parameters; L. C., B. F. and Z. F. determined the gene and protein expressions; D. W., J. W. and M. C. carried out data analyses; D. W., S. X. and B. F. made modifications to the manuscript. All the authors read and approved the final version of the manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/doi:10.1017/S0007114516002166>

Reference

- Lam DW & LeRoith D (2012) The worldwide diabetes epidemic. *Curr Opin Endocrinol Diabetes Obes* **19**, 93–96.
- Duque-Guimarães DE & Ozanne SE (2013) Nutritional programming of insulin resistance: causes and consequences. *Trends Endocrinol Metabol* **24**, 525–535.
- Giussani DA (2011) The vulnerable developing brain. *Proc Natl Acad Sci* **108**, 2641–2642.
- Barker D (2012) Developmental origins of chronic disease. *Public Health* **126**, 185–189.
- Latouche C, Heywood SE, Henry SL, *et al.* (2014) Maternal overnutrition programs changes in the expression of skeletal muscle genes that are associated with insulin resistance and defects of oxidative phosphorylation in adult male rat offspring. *J Nutr* **144**, 237–244.
- Lie S, Morrison JL, Williams-Wyss O, *et al.* (2014) Periconceptional undernutrition programs changes in insulin signaling molecules and MicroRNAs in skeletal muscle in singleton and twin fetal sheep. *Biol Reprod* **90**, 5.
- Samuel VT & Shulman GI (2012) Mechanisms for insulin resistance: common threads and missing links. *Cell* **148**, 852–871.
- Jornayvaz FR, Samuel VT & Shulman GI (2010) The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. *Annu Rev Nutr* **30**, 273–290.
- Zorzano A, Palacin M & Guma A (2005) Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. *Acta Physiol Scand* **183**, 43–58.
- Vogt C, Ardehali H, Iozzo P, *et al.* (2000) Regulation of hexokinase II expression in human skeletal muscle *in vivo*. *Metabolism* **49**, 814–818.
- Kruszynska YT, Ciaraldi TP & Henry RR (2011) Regulation of glucose metabolism in skeletal muscle. *Compr Physiol* **7**, 579–607.
- Liu J (2012) Intrauterine growth retardation increases the susceptibility of pigs to high-fat diet-induced mitochondrial dysfunction in skeletal muscle. *PLOS ONE* **7**, e34835.
- Zheng S, Rollet M & Pan Y-X (2012) Protein restriction during gestation alters histone modifications at the glucose transporter 4 (GLUT4) promoter region and induces GLUT4 expression in skeletal muscle of female rat offspring. *J Nutr Biochem* **23**, 1064–1071.
- De Blasio MJ, Gattford KL, Harland ML, *et al.* (2012) Placental restriction reduces insulin sensitivity and expression of insulin signaling and glucose transporter genes in skeletal muscle, but not liver, in young sheep. *Endocrinology* **153**, 2142–2151.
- Thamotharan M, Shin BC, Suddiricku DT, *et al.* (2005) GLUT4 expression and subcellular localization in the intrauterine growth-restricted adult rat female offspring. *Am J Physiol Endocrinol Metab* **288**, 935–947.
- Muhlhauser BS, Duffield JA, Ozanne SE, *et al.* (2009) The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. *J Physiol* **587**, 4199–4211.
- Gavete ML, Martín MA, Alvarez C, *et al.* (2005) Maternal food restriction enhances insulin-induced GLUT-4 translocation and insulin signaling pathway in skeletal muscle from suckling rats. *Endocrinology* **146**, 3368–3378.
- Devaskar SU & Chu A (2016) Intrauterine growth restriction: hungry for an answer. *Physiology* **31**, 131–146.
- Thorn SR, Rozance PJ, Brown LD, *et al.* (2011) The intrauterine growth restriction phenotype: fetal adaptations and potential implications for later life insulin resistance and diabetes. *Semin Reprod Med* **29**, 225–236.
- Wang J, Wu Z, Li D, *et al.* (2012) Nutrition, epigenetics, and metabolic syndrome. *Antioxid Redox Signal* **17**, 282–301.
- Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* **13**, 484–492.
- Raychaudhuri N, Raychaudhuri S, Thamotharan M, *et al.* (2008) Histone code modifications repress glucose transporter 4 expression in the intrauterine growth-restricted offspring. *J Biol Chem* **283**, 13611–13626.
- Miller E & Ullrey D (1987) The pig as a model for human nutrition. *Annu Rev Nutr* **7**, 361–382.
- Neeb ZP, Edwards JM, Alloosh M, *et al.* (2010) Metabolic syndrome and coronary artery disease in Ossabaw compared with Yucatan swine. *Comparative medicine* **60**, 300–315.
- Poore K & Fowden A (2002) The effect of birth weight on glucose tolerance in pigs at 3 and 12 months of age. *Diabetologia* **45**, 1247–1254.
- Poore K & Fowden A (2004) Insulin sensitivity in juvenile and adult large white pigs of low and high birthweight. *Diabetologia* **47**, 340–348.
- Guan X, Matte JJ, Ku PK, *et al.* (2000) High chromium yeast supplementation improves glucose tolerance in pigs by decreasing hepatic extraction of insulin. *J Nutr* **130**, 1274–1279.
- Rosenfold K, Petersen J, Lærke H, *et al.* (2001) Muscle glycogen stores and meat quality as affected by strategic finishing feeding of slaughter pigs. *J Anim Sci* **79**, 382–391.
- Petry CJ, Dorling MW, Pawlak DB, *et al.* (2001) Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int J Exp Diabetes Res* **2**, 139–143.
- Ford S, Hess B, Schwoppe M, *et al.* (2007) Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *J Anim Sci* **85**, 1285–1294.

31. Li Y, He Y, Qi L, *et al.* (2010) Exposure to the Chinese famine in early life and the risk of hyperglycemia and type 2 diabetes in adulthood. *Diabetes* **59**, 2400–2406.
32. Lim K, Armitage JA, Stefanidis A, *et al.* (2011) IUGR in the absence of postnatal 'catch-up' growth leads to improved whole body insulin sensitivity in rat offspring. *Pediatr Res* **70**, 339–344.
33. Limesand SW, Rozance PJ, Smith D, *et al.* (2007) Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction. *Am J Physiol Endocrinol Metab* **293**, 1716–1725.
34. Owens JA, Gatford KL, Blasio MJD, *et al.* (2007) Restriction of placental growth in sheep impairs insulin secretion but not sensitivity before birth. *J Physiol* **584**, 935–949.
35. Iniguez G, Ong K, Bazaes R, *et al.* (2006) Longitudinal changes in insulin-like growth factor-I, insulin sensitivity, and secretion from birth to age three years in small-for-gestational-age children. *J Clin Endocrinol Metab* **91**, 4645–4649.
36. Mericq V, Ong KK, Bazaes R, *et al.* (2006) Longitudinal changes in insulin sensitivity and secretion from birth to age three years in small- and appropriate-for-gestational-age children. *Diabetologia* **48**, 2609–2614.
37. Kim JK, Zisman A, Fillmore JJ, *et al.* (2001) Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin Invest* **108**, 153–160.
38. Zisman A, Peroni OD, Abel ED, *et al.* (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* **6**, 924–928.
39. Ozanne SE, Wang CL, Coleman N, *et al.* (1997) Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *Am J Physiol* **271**, 1128–1134.
40. Agote M, Goya L, Ramos S, *et al.* (2001) Glucose uptake and glucose transporter proteins in skeletal muscle from undernourished rats. *Am J Physiol Endocrinol Metab* **281**, 1101–1109.
41. Freitas MSD, Souza EPGD, Silva SVD, *et al.* (2003) Up-regulation of phosphatidylinositol 3-kinase and glucose transporter 4 in muscle of rats subjected to maternal under-nutrition. *Biochim Biophys Acta* **1639**, 8–16.
42. Raychaudhuri N, Raychaudhuri S & Devaskar S (2008) Histone code modifications repress glucose transporter 4 expression in the intrauterine growth-restricted offspring. *J Biol Chem* **283**, 13611–13626.
43. Ozanne SE, Jensen CB, Tingey KJ, *et al.* (2005) Low birth-weight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia* **48**, 547–552.
44. Jensen CB, Martin-Gronert MS, Storgaard H, *et al.* (2008) Altered PI3-kinase/Akt signalling in skeletal muscle of young men with low birth weight. *PLoS ONE* **3**, e3738.
45. Lillycrop KA & Burdge GC (2012) Epigenetic mechanisms linking early nutrition to long term health. *Best Pract Res Clin Endocrinol Metab* **26**, 667–676.
46. Raynal NJ-M, Si J, Taby RF, *et al.* (2012) DNA methylation does not stably lock gene expression but instead serves as a molecular mark for gene silencing memory. *Cancer Res* **72**, 1170–1181.
47. Cedar H & Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* **10**, 295–304.
48. Calvanese V, Lara E, Kahn A, *et al.* (2009) The role of epigenetics in aging and age-related diseases. *Ageing Res Rev* **8**, 268–276.
49. Benayoun BA, Pollina EA & Anne B (2015) Epigenetic regulation of ageing: linking environmental inputs to genomic stability. *Nat Rev Mol Cell Biol* **16**, 593–610.
50. Park JH, Stoffers DA, Nicholls RD, *et al.* (2008) Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. *J Clin Invest* **118**, 2316–2324.
51. Sandovici I, Smith NH, Nitert MD, *et al.* (2011) Maternal diet and aging alter the epigenetic control of a promoter-enhancer interaction at the Hnf4a gene in rat pancreatic islets. *Proc Natl Acad Sci* **108**, 5449–5454.
52. Murgatroyd C, Patchev AV, Wu Y, *et al.* (2009) Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat Neurosci* **12**, 1559–1566.
53. Portela A & Esteller M (2010) Epigenetic modifications and human disease. *Nat Biotechnol* **28**, 1057–1068.
54. Vestergaard H, Bjørbaek C, Hansen T, *et al.* (1995) Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients. *J Clin Invest* **96**, 2639–2645.
55. Bouskila M, Hunter RW, Ibrahim AF, *et al.* (2010) Allosteric regulation of glycogen synthase controls glycogen synthesis in muscle. *Cell Metab* **12**, 456–466.
56. Petersen KF & Shulman GI (2002) Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. *Am J Cardiol* **90**, 11–18.
57. Chaneton B & Gottlieb E (2012) Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. *Trends Biochem Sci* **37**, 309–316.
58. Schroeder MA, Atherton HJ, Dodd MS, *et al.* (2012) The cycling of acetyl-coenzyme A through acetylcarnitine buffers cardiac substrate supply: a hyperpolarized ¹³c magnetic resonance study. *Circ Cardiovasc Imaging* **5**, 201–209.
59. Pan-Zhou XR, Cui L, Zhou XJ, *et al.* (2000) Differential effects of antiretroviral nucleoside analogs on mitochondrial function in HepG2 cells. *Antimicrob Agents Chemother* **44**, 496–503.
60. Long YC, Kostovski E, Boon H, *et al.* (2011) Differential expression of metabolic genes essential for glucose and lipid metabolism in skeletal muscle from spinal cord injured subjects. *J Appl Physiol* **110**, 1204–1210.
61. Choi CS, Kim YB, Lee FN, *et al.* (2002) Lactate induces insulin resistance in skeletal muscle by suppressing glycolysis and impairing insulin signaling. *Am J Physiol Endocrinol Metab* **283**, 233–240.
62. Spoden GA, Rostek U, Lechner S, *et al.* (2009) Pyruvate kinase isoenzyme M2 is a glycolytic sensor differentially regulating cell proliferation, cell size and apoptotic cell death dependent on glucose supply. *Exp Cell Res* **315**, 2765–2774.
63. Saltiel AR & Kahn CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799–806.
64. Thom SR, Regnault TR & Brown LD (2009) Intrauterine growth restriction increases fetal hepatic gluconeogenic capacity and reduces messenger ribonucleic acid translation initiation and nutrient sensing in fetal liver and skeletal muscle. *Endocrinology* **150**, 3021–3030.
65. Oak SA, Tran C, Pan G, *et al.* (2006) Perturbed skeletal muscle insulin signaling in the adult female intrauterine growth-restricted rat. *Am J Physiol Endocrinol Metab* **290**, 1321–1330.