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Maternal low-protein diet causes epigenetic deregulation of HMGCR and CYP7α1 in the liver of weaning piglets☆

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Abstract

To investigate the effect of maternal dietary protein on hepatic cholesterol metabolism in offspring pigs and to detect underlying epigenetic mechanisms, 14 primiparous purebred Meishan sows were fed standard-protein (SP, $n=7$) or low-protein (LP, 50% of SP, $n=7$) diets during pregnancy and lactation, respectively. LP piglets showed significantly lower body weight and liver weight at weaning, associated with decreased liver and serum cholesterol content. Hepatic *SREBP2*, *HMGCR* and *CYP7α1* mRNA expressions were all up-regulated in LP piglets, as well as SREBP2 protein content and HMGCR enzyme activity, compared to SP piglets, while the mRNA expression of *LDLR, FXR, LXR* and *CYP27α1* was not altered. Hepatic activation of *HMGCR* gene transcription in LP piglets was associated with promoter hypomethylation, together with decreased histone H3, H3 lysine 9 monomethylation (H3K9me1) and H3 lysine 27 trimethylation (H3K27me3) and increased H3 acetylation. No CpG islands were predicted in the *CYP7α1* promoter, and the augmented *CYP7α1* transcription in LP piglets was associated with decreased H3, H3K9me1 and H3K27me3. No alterations were detected for hepatic expression of microRNAs predicted to target 3′-UTR of *HMGCR* or *CYP7α1* gene. These results indicate that maternal low-protein diet during gestation and lactation affects hepatic cholesterol metabolism in weaning piglets by modifying the epigenetic regulation of *HMGCR* and *CYP7α1* genes, which implicates possible long-term consequences in cholesterol homeostasis later in adult life.

Keywords: Maternal dietary protein; Meishan piglets; Liver; DNA methylation; Histone modification; Cholesterol metabolism

1. Introduction

Epidemiological and experimental studies indicate that maternal malnutrition during gestation and lactation leads to lower birth weight and deregulation of metabolic homeostasis in offspring [1]. A number of studies on rodents have demonstrated the effects of maternal protein restriction or low-protein (LP) diet on offspring cholesterol homeostasis [2,3]. However, results from different laboratories are inconsistent, with reports of increase [4,5], decrease [3,6–8] or no change [2,9] in plasma cholesterol levels in offspring derived from dams fed LP diet (40%–50% of control). As plasma cholesterol level is highly variable with numerous interfering factors, future mechanistic studies are directed to focus on the liver [3].

Liver is a target for maternal nutritional programming [10] and plays an important role in the regulation of cholesterol homeostasis [11]. So far, there are only few publications describing the effects of protein restriction during gestation and/or lactation on offspring hepatic cholesterol homeostasis [3,4,12]. Similar to the plasma cholesterol concentration, the responses of the hepatic cholesterol content differ in different studies. While an LP diet (8% vs. 19%) throughout pregnancy and lactation causes decreased hepatic cholesterol content in 150-day-old SD rat offspring [3], a similarly designed experiment using Wistar rats demonstrated opposite result, showing higher hepatic cholesterol content in 130-day-old male offspring derived from dams fed LP diet (8% vs. 20%) during gestation and lactation [4]. Besides these inconsistencies, little is known about

Abbreviations: CP, crude protein; CYP7α1, cholesterol-7alpha-hydroxylase; FXR, farnesoid X receptor; H3ac, histone H3 acetylation; H3K9me1, histone H3 lysine 9 monomethylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K27me3, histone H3 lysine 27 trimethylation; HDLC, high-density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLC, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LP, low protein; LXR, liver X-activated receptor; SP, standard protein; SREBP, sterol regulator element-binding protein; Tch, total cholesterol.

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the molecular mechanisms underlying the effects of maternal dietary protein on offspring hepatic cholesterol homeostasis.

Hepatic cholesterol content is determined by a net balance of de novo cholesterol biosynthesis, cholesterol transport and catabolism, i.e., the conversion of cholesterol to bile acids [13]. 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is the rate-limiting and irreversible step in cholesterol biosynthesis [14]. Transcriptional regulation of *HMGCR* is governed by a family of sterol regulatory element binding proteins (SREBPs). SREBP2 is the predominant isoform in the liver exhibiting preference in regulating cholesterol homeostasis [15,16]. The activity of both HMGCR and SREBP2 is negatively regulated by the intracellular cholesterol, forming a feedback loop for cholesterol homeostasis [17]. Cholesterol transport refers to the dynamic equilibrium of cholesterol influx and efflux in the liver. Low-density lipoprotein (LDL) molecules are the major carriers of cholesterol in the blood which are responsible for secretion of cholesterol of liver to other peripheral tissues, whereas high-density lipoproteins (HDLs) mediate the reverse cholesterol transport from the extrahepatic tissues back to the liver. The ratio of LDL-cholesterol (LDLC) to HDL-cholesterol (HDLC) provides a crude estimation of the dynamic equilibrium. Low-density lipoprotein receptor (LDLR) mediates the uptake of LDLC, thus playing an important role in regulating serum concentrations of total cholesterol (Tch) and LDLC. Cholesterol-7alpha-hydroxylase (CYP7α1) is the rate-limiting enzyme for the predominant pathway of bile acid synthesis from cholesterol in the liver [18], while cholesterol-27alpha-hydroxylase (CYP27α1) is responsible for the alternative pathway of bile acid synthesis. The liver X receptor (LXR) and farnesoid X receptor (FXR) are nuclear receptors which regulate a number of target genes that are crucially involved in cellular lipid homeostasis, including LDLR, CYP7α1, etc. [19].

A number of studies on rodents indicate that maternal dietary protein influences hepatic lipid or cholesterol metabolism in offspring through epigenetic mechanisms including DNA methylation, histone modification and microRNA-mediated translational repression and gene silencing. In a series of experiments, Lillycrop and colleagues demonstrated that maternal dietary protein restriction modifies the pattern of expression and promoter methylation of genes involved in metabolic processes in the liver [20–23]. *SREBP*s, *HMGCR*, *LXR* and *CYP7α1* have been identified as target genes affected by maternal nutrition to mediate the fetal programming of offspring lipid metabolism in the liver. For instance, maternal hypercholesterolemia causes transcriptional activation of genes involved in endogenous cholesterol synthesis, including SREBP2 and HMGCR, in the liver of adult mice offspring [24]. Maternal low-protein diet affects hepatic cholesterol homeostasis by altering histone modifications at *CYP7α1* promoter in rat offspring [4]. Prenatal protein restriction decreases cholesterol content in fetal liver, which is associated with hyper-methylated *LXR* gene promoter in mice [8].

A number of miRNAs (miRs) have been identified to execute posttranscriptional regulation of genes involved in lipid homeostasis [25,26]. Recently, miR-33 located in the intron of *SREBP*s has been reported to regulate cholesterol metabolism [27,28]. However, it remains unknown whether the programming effect of maternal low-protein diet on offspring hepatic cholesterol homeostasis may involve miRNA-mediated posttranscriptional regulation.

Pigs serve as an ideal model for metabolic studies because they are similar to humans in phenotypes like cardiovascular anatomy and function, metabolism, lipoprotein profile and omnivorous habits [29,30]. However, studies on fetal programming of hepatic cholesterol homeostasis in the pig are missing.

Meishan (MS), a Chinese indigenous pig breed, is traditionally raised on low-protein diets containing approximately half the amount of crude protein in the modern commercial diets. In the present study, we used MS pig as model to delineate the impact of maternal dietary protein on hepatic cholesterol metabolism in offspring piglets at weaning. MS sows were fed diets containing either low-protein as in traditional diet or standard-protein as in modern diet throughout gestation and lactation. Blood and liver samples of the offspring piglets were taken at weaning on 35 d of age to determine the profiles of cholesterol metabolism and expression of relevant genes, together with the associated alterations in epigenetic modification on the promoter of affected genes.

2 Materials and methods

2.1 Animal and sampling

The animal experiment was conducted in the National Meishan Pig Preservation and Breeding Farm at Jiangsu Polytechnic College of Agriculture and Forestry, Jurong, Jiangsu Province, P.R. China. Fourteen primiparous purebred MS gilts (body weight: 36.1±1.8 kg) were assigned randomly into standard- (SP) and low- (LP) protein groups. The SP sows were fed on diets containing 12% and 14% crude protein (CP), while LP sows were fed on diets containing 6% and 7% CP, during gestation and lactation, respectively (Table 1). Litter size was adjusted to seven to eight pigs per litter at 24 h postfarrowing in the same group. Newborn piglets were allowed free access to their mothers until 35 days of age when male piglets (one per litter) were weighted and killed for sampling. Serum samples were prepared and stored at −20°C, and liver samples were taken within 20 min postmortem, snap-frozen in liquid nitrogen and stored at −80°C until further analysis (Table 2).

The animal experiment was undertaken following the guidelines of the Animal Ethics Committee of Nanjing Agricultural University, China.

2.2 Cholesterol in serum and liver

Total cholesterol in serum and liver was measured using a commercial cholesterol assay kit (AO10027, Jinma Biotechnology Co., Ltd., Wenzhou, China). Serum concentrations of LDLC and HDLC were measured with respective assay kits (006340 and 006328, respectively, Beijing BHKT Clinical Reagent Co., Ltd., Beijing, China). The cholesterol in liver was extracted according to a previously reported procedure [31]. Briefly, 200 mg frozen liver sample was homogenized in 1 ml of lysis buffer (18 mmol/L Tris, pH 7.5, 300 mmol/L mannitol, 50 mmol/L EDTA, 0.1 mmol/L PMSF) by a Polytron homogenizer (PT1200E, Brinkman Instruments, Littau, Switzerland). Two hundred microliters of homogenate was vigorously mixed with 800 μl chloroform/methanol (2:1, vol/vol) and centrifuged at 3000*g* for 5 min. The cholesterol in the organic phase was extracted, air-dried and reconstituted in 30-μl mixture of *tert*-butyl alcohol and methanol (13:2, vol/vol). The cholesterol content was determined by cholesterol assay kit (Jinma Biotechnology Co., Ltd., Wenzhou, China).

Table 1
Composition and nutrient content of the experimental diet

	Gestation period		Lactation period	
	SP	LP	SP	LP
Ingredient, %				
Corn	58.0	52.8	61.0	55.8
Soybean meal	12		17	
Bran	15	11	12	15
Bone meal	1	0.5	1	0.5
Corn sugar	10	27	5	22
CaSPO4		0.7		0.7
Fiber[a]		1		1
Attapulgite		3		1
Premix[b]	4	4	4	4
Calculated composition				
Digestible energy, MJ/kg	13.1	13.1	13.1	13.1
Crude protein, %	12.1	6.1	14	6.9
Crude fiber, %	2.7	2.3	2.8	2.6
Ca, %	1.2	1.2	1.2	1.2
P, %	0.4	0.4	0.4	0.4

[a] The fiber concentrate ARBOCEL was purchased from JRS (Germany).

[b] The premix contains (per kilogram): vitamin A: 240,000 IU; vitamin D3: 60,000 IU; vitamin E: 720 IU; vitamin K3: 30 mg; vitamin B1: 30 mg; vitamin B2: 120 mg; vitamin B6: 60 mg; vitamin B12: 360 mg; niacin: 600 mg; pantothenic acid: 300 mg; folic acid: 6 mg; manganese sulphate: 1.0 g; zinc oxide: 2.5 g; iron sulphate: 4.0 g; copper sulphate: 4.0 g; sodium selenite: 6 mg; calcium: 150 g; phosphorus: 15 g; sodium chloride: 40 g.

Table 2
Body weight, liver weight and the content of cholesterol in plasma and liver of the offspring piglets at weaning

Parameters	SP	LP	*P* value
Body weight (kg)	6.62±0.46	5.14±0.39 *	.03
Liver weight (g)	172.50±7.25	135.27±6.35 **	.00
Liver index (g/kg)	26.59±1.52	26.82±1.22	.91
Serum Tch (mmol/L)	3.46±0.38	2.41±0.17 *	.03
LDLC (mmol/L)	1.78±0.34	1.12±0.37	.22
HDLC (mmol/L)	0.97±0.06	0.78±0.10	.12
LDLC/HDLC	1.95±0.48	1.79±0.68	.85
Tch (mg/g liver)	2.00±0.08	1.76±0.04 *	.02

Values are means±S.E.M., $n=7$.
* $P<.05$.
** $P<.01$ compared to SP.

2.3. Total RNA isolation and mRNA quantification

Total RNA was isolated from liver samples using Trizol Reagent (Tiangen Biotech Co., Ltd., Beijing, China). The iScript cDNA Synthesis Kit (Promega, Madison, WI, USA) was used to synthesize cDNA from 2 μg of total RNA from each sample according to the manufacturer's instructions. Two microliters of diluted cDNA (1:50) was used in real-time polymerase chain reaction (PCR). Primer sequences are shown in Table 3 and were synthesized by Invitrogen (Shanghai, China). Real-time PCR was performed in Mx3000P (Stratagene, USA). As *GAPDH* mRNA abundance in liver was unaffected by maternal dietary treatment, the technical variations were normalized with *GAPDH* as internal control. Mock reverse transcription (RT) and no template controls were set to monitor the possible contamination of genomic and exogenous DNA both at RT and PCR. The specificity of amplification was determined by melting curve analysis and PCR product sequencing.

2.4. Quantitation of miRNAs targeting HMGCR and CYP7α1

Total RNA was treated with RNase-free DNase I (Takara, Biotechnology, China), and 2 μg of treated total RNA was polyadenylated by poly (A) polymerase at 37°C for 1 h in a 20-μl reaction mixture using Poly (A) Tailing Kit (AM1350, Applied Biosystems, USA) according to the manufacturer's instructions. Treated RNA was then dissolved and reverse transcribed using poly (T) adapter. Real-time PCR was performed with SYBR green qPCR master mix reagent (TaKaRa, Japan) in triplicates with a miRNA specific forward primer and a universal reverse primer complementary to part of the poly (T) adapter sequence. Since no validated reference gene was available for pig miRNA, a random DNA oligonucleotide was added to RNase-free DNase I treated total RNA samples before polyadenylation, as an exogenous reference, to normalize the expression of miRNAs. The sequences of all the primers, poly (T) adapter and exogenous reference used are listed in Table 4.

2.5. Tissue protein extraction and Western blot analysis

Total cellular protein and nuclear protein were extracted from 100-mg frozen liver tissue as described previously [32]. The protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Western blot analysis for CYP7α1 (ab79847, Abcam, UK, diluted 1:100) and SREBP2 (ab30682, Abcam, UK, diluted 1:200) was carried out according to the recommended protocols provided by the manufacturers. The CYP7α1 protein content in total cellular lysates was normalized with GAPDH (KC-5G4, Kangcheng, China, diluted 1:10,000). SREBP2 content in the nuclei was normalized with histone H1 (BS1165, Bioworld Technology, USA, diluted 1:1000).

Table 3
Nucleotide sequences of specific primers

Target genes	Sequence (5′to 3′)	Products (bp)	GenBank no.
SREBP2	F: GCCTACCGCAAGGTGTTTC R: GTCATTTGCTGGCAGTCGTT	305	DQ020476.1
HMGCR	F: CAGGCTGAAGTAAGGGAGA R: CACGAAGTAGGTGGCGAGA	174	DQ432054.1
LDLR	F:ACTGCTCATCCTCCTCTT R:TTCCGTGGTCTTCTGGTA	109	AF065990.1
LXR	F: ATTTCCAGGAGTGCCGTCTT R: CTTGCCGCTTCAGTTTCTT	102	AB254406.1
FXR	F: CGGAGAAGCATTACCA R: AAGCATTCAGCCAACA	137	XM 001928800.2
CYP7α1	F: TATTCCTTCCGTTACCGAGTG R: ACCTGACCAGTTCCGAGAT	262	AK230868.1
CYP27a1	F: TGTGGCTCGCATCGTTC R:TCACCTGGCAGCTCCTT	153	EF625352.1
GAPDH	F: TACATGGTCTACATGTTCCAGTATG R: CAGGAGGCATTGCTGACAATCTTG	285	DQ403065
HMGCR promoter	F: CCCCTATCGTCTTCGC R: CTTGTCCCGCACCATCT	133	EU729728.1
CYP7α1 promoter	F: AGACGGGAGGGTCAGA R:GCCAGTGGGATGTAATGT	149	NC_010446

2.6. HMGCR activity assay

HMGCR activity in liver lysates was measured with HMGCR Activity Assay Kit (CS1090, Sigma, USA) according to manufacturer's instructions. The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGR in the presence of the substrate HMG-CoA. Liver tissue was homogenated in HMGCR assay buffer (pH 7.1) containing 100 mM phosphate buffer (KH_2PO_4, Na_2HPO_4, pH 7.5), 200 mM KCl, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, 1 μg/ml aprotinin and 0.5 μg/ml leupeptin (1:3, wt/vol) on ice for 5 min, and then centrifuged for 5 min at 1000*g* at 4°C. Protein concentration was determined with Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Twenty microliters of supernatant was taken to measure HMGCR activity. One unit converts 1.0 μmol of NADPH to $NADP^+$ per 1 min at 37°C. The unit specific activity is defined as μmol/min/mg protein (units/mg P).

2.7. DNA methylation detection — MeDIP

High-quality genomic DNA isolated from liver tissues was sonicated to produce small fragments (300–1000 bp). Two micrograms of fragmented DNA was heat denatured to produce single-stranded DNA, and a portion of the denatured DNA was stored as control (input) DNA. Mouse monoclonal antibody against 5-methyl cytidine (ab10805, Abcam, UK) was used to immunoprecipitate methylated DNA fragments. The immune complexes were captured with protein G agarose beads (80 μl, 50% slurry, pretreated with denatured salmon sperm DNA and BSA, Biyuntian, Biotechnology, China). The beads bound with immune complexes were washed to remove nonspecific binding and resuspended in 250 μl digestion buffer containing proteinase K. The MeDIP DNA was phenol extracted and ethanol precipitated. A small aliquot of MeDIP DNA and of control input DNA was used to amplify the *HMGCR* proximal promoter sequences by real-time PCR with specific primers designed with Primer 5 software (Table 3). The CpG islands on the promoters of the candidate genes were assessed by Methyl Primer Express v1.0 (Applied Biosystems, USA) using the following criteria: %GC >50%, length >200 bp, CpG observed/CpG expected >0.6. No CpG islands were found within 5′ flanking sequence (up to 30,000 bp) of the porcine *CYP7α1* gene (ENSSSCT00000006836, F1RT71_PIG). Since there was a sequence gap located in the proximal 5′ flanking sequence, we used the sequence of 1027 bp which is right after the gap for further assessment; only nine CpG sites were found, and no CpG islands were predicted within this sequence. Therefore, we excluded MeDIP analysis for *CYP7α1* promoter in the present study.

2.8. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to the previous publications with some modifications [33,34]. Briefy, 200-mg frozen liver samples were ground in liquid nitrogen, resuspended in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and cross-linked in 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped using 2.5 M glycine while rotating for 10 min at room temperature. The pellets were washed using PBS and rinsed with sodium dodecyl sulfate (SDS) lysis buffer (50 M Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. Cross-linked samples were sonicated for 10 min on ice with 10-s on/off intervals (Sonics Vibra, USA). The samples were then centrifuged at 12,000 rpm for 10 min at 4°C to remove cell

Table 4
miRNA and the corresponding primer sequences

Name	Sequence (5′to 3′)	miRbase accession
ssc-miR-23a	ATCACATTGCCAGGGATTTCC	MIMAT0002133
ssc-miR-23b	ATCACATTGCCAGGGATTACCA A	MIMAT0013893
ssc-miR-29c	TAGCACCATTTGAAATCGGTTA	MIMAT0002166
ssc-miR-497	CAGCAGCACACTGTGGTTTGT	MIMAT0013926
ssc-miR-145	GTCCAGTTTTCCCAGGAATCCCTT	MIMAT0002123
ssc-miR-181c	AACATTCAACCTGTCGGTGAGT	MIMAT0002143
ssc-miR-221	AGCTACATTGTCTGCTGGGTTT	MIMAT0007762
ssc-miR-499-5p	TTAAGACTTGCAGTGATGTTT	MIMAT0013877
ssc-miR-15b	TCGAGGAGCTCACAGTCTAGT	MIMAT0013882
Oligo dT adaptor	TAGAGTGAGTGTAGCGAGCACAGAA TTAATACGACTCACTATAGGTTTTTT TTTTTTTTTVN	N/A
Universal primer	TAGAGTGAGTGTAGCGAGCA	N/A
Exogenous reference	GTGACCCACGATGTGTATTCGC	

debris from the crude chromatin preparations. The average length of sonicated chromatin was around 500 bp determined by resolving on a 1% agarose gel. Protein–DNA complex was diluted in ChIP dilution buffer, precleared with salmon sperm DNA/protein G agarose beads (60 μl, 50% slurry, Biyuntian, Biotechnology, China), incubated with 2 μg of respective antibody [histone H3 antibody, ab1791, Abcam; anti-acetyl-histone H3, 06-599, Millipore; monomethyl-Histone H3K9 (Lys9) 17-680, Millipore; trimethyl-histone H3K27 (Lys27), 17-622, Millipore; trimethyl-histone H3K4 antibody, ab1012, Abcam] overnight at 4°C. A negative control was included without adding antibody. Protein G agarose beads (120 μl, 50% slurry) were added to capture the immunoprecipitated chromatin complexes. The pellets containing immunoprecipitated complexes were sequentially washed, and the antibody/protein/DNA complexes were eluted from protein G agarose beads. Finally, reverse cross-linking was performed to release DNA fragments from the immunoprecipitated complex at 65°C for 5 h, and DNA was purified. Immunoprecipitated DNA was used as template for real-time PCR using specific primers to amplify genomic sequences at the promoter region of *HMGCR* and *CYP7α1* genes (Table 3). No genomic DNA sequence is available for *SREBP2*, so ChIP-qPCR assay for *SREBP2* promoter was excluded from the present study.

2.9. Statistical analysis

All data are presented as means±S.E.M. and were analyzed using independent-samples *t* test with SPSS 13.0 for windows. The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data expressed as the fold change relative to the SP group. Differences were considered statistically significant when $P<.05$.

3. Results

3.1. Body weight and cholesterol in serum and liver

As shown in Table 2, piglets derived from LP sows exhibited significantly lower body weight ($P<.05$) and liver weight ($P<.01$) at weaning, yet the relative liver weight to body weight (liver index) was unchanged. This growth retardation is associated with significantly decreased serum concentration and liver content of Tch ($P<.05$). Maternal LP diet did not affect serum concentrations of LDLC, HDLC or the ratio of LDLC to HDLC.

3.2. Hepatic expression of genes involved in cholesterol metabolism

The mRNA abundance of seven genes involved in hepatic cholesterol metabolism was quantitated with real-time RT-PCR. Among these genes, two genes responsible for cholesterol biosynthesis, *SREBP2* and *HMGCR*, and one gene involved in cholesterol catabolism, *CYP7α1*, were found to be significantly up-regulated in the liver of LP piglets (Fig. 1, $P<.05$).

3.3. Hepatic SREBP2 and CYP7α1 protein content and HMGCR activity

Hepatic protein content of SREBP2 and CYP7α1 was determined with Western blot analysis. In line with hepatic mRNA abundance, SREBP2 content in nuclear lysates was significantly higher (Fig. 2A, $P<.05$) in the liver of LP piglets. However, CYP7α1 protein content in the whole tissue lysates was not affected by maternal dietary protein level (Fig. 2B), although CYP7α1 mRNA expression was significantly up-regulated in the liver of LP piglets.

In agreement with mRNA expression, HMGCR activity in liver lysates was significantly increased in weaning piglets derived from sows fed on LP diets (Fig. 2C, $P<.05$).

3.4. Expression of microRNAs predicted to target HMGCR and CYP7α1

Two algorithms, Probability of Interaction by Target Accessibility (PITA) [35] and RNA22 [36], were applied to predict the miRNAs targeting 3′ UTR of *HMGCR* and *CYP7α1*. Twelve miRNAs for *HMGCR* and 10 for *CYP7α1*, which are on the top of intersection of PITA and RNA22, were chosen for miRNA quantification. Real-time quantitative RT-PCR detected four miRNAs targeting *HMGCR* and five targeting *CYP7α1* (Table 5). None of the miRNAs was differentially expressed between the LP and SP groups.

3.5. DNA methylation and histone modifications

MeDIP analysis revealed significant hypomethylation ($P<.01$) of *HMGCR* promoter in the liver of LP piglets (Fig. 3), corresponding to the augmented *HMGCR* mRNA expression. As no CpG islands are predicted to exist in the *CYP7α1* promoter, MeDIP analysis for *CYP7α1* promoter was excluded from the present study.

The enrichment of four histone modification marks, namely, histone H3 acetylation (H3ac), histone H3 lysine 9 monomethylation (H3K9me1), histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 trimethylation (H3K27me3), as well as histone H3 on the promoter of *HMGCR* and *CYP7α1*, was determined with ChIP assay using specific antibodies. As shown in Fig. 4A, hepatic activation of *HMGCR* gene transcription in LP piglets was associated with a 60.6% increase in H3ac ($P<.05$) and significant decrease ($P<.05$) in histone H3 (−56.8%), H3K9me1 (−67.6%) and H3K27me3 (−83.1%), expressed as percentage of the input. When expressed as the ratio relative to H3, a 157.0% increase in H3ac ($P<.01$), a 67.8% decrease in H3K9me1 ($P<.01$) and a 62.3% decrease in H3K27me3 ($P=.08$) were detected at the *HMGCR* promotor in LP piglets, corresponding to the 131.5% increase in *HMGCR* mRNA (Fig. 4B).

Fig. 4C shows the enrichment of histone H3 and the modified forms at the *CYP7α1* promoter, expressed as percentages of the input. The 242.9% of increase in *CYP7α1* transcription in LP piglets was associated with a trend of decrease ($P=.07$) in total H3 (−46.7%) and significantly ($P<.05$) decreased H3K9me1 (−86.9%) and H3K27me3 (−69.6%), expressed as percentage of the input. A significant decrease of H3K9me1 (−74.6%, $P<.05$) and a trend of increase of H3K4me3 (188.7%, $P=.06$) were seen at the *CYP7α1* promoter in LP piglets when expressed as the ratio to total H3 (Fig. 4D). No difference in H3K4me3 was detected at the promoter of either *HMGCR* or *CYP7α1* when expressed as percentage to the input.

4. Discussion

In this study, we present evidence that an LP diet during pregnancy and lactation led to lower body weight and liver weight in male weaning piglets at postnatal day 35. Our results agree with the previous reports that lower serum cholesterol is accompanied by significantly lower birth weight and weaning weight, as well as slower growth rate in pigs [37,38]. LDLC/HDLC ratio is regarded as a reliable measure for cholesterol homeostasis. High LDLC/HDLC ratio is associated with high risk for cardiovascular diseases. In this study, although LP piglets had 37.1% lower LDLC and 19.6% lower

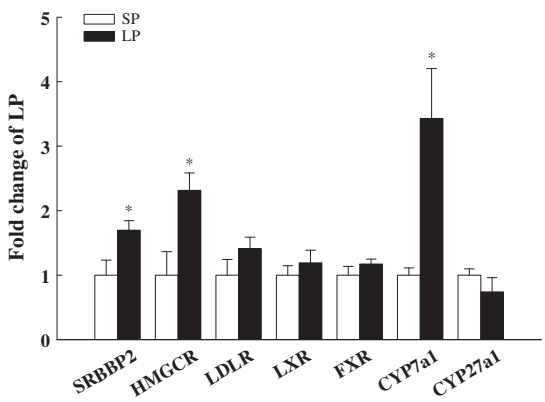

Fig. 1. Hepatic expression of genes involved in cholesterol metabolism in weaning piglets. Values are means±S.E.M., $n=7$. *$P<.05$ compared with SP.

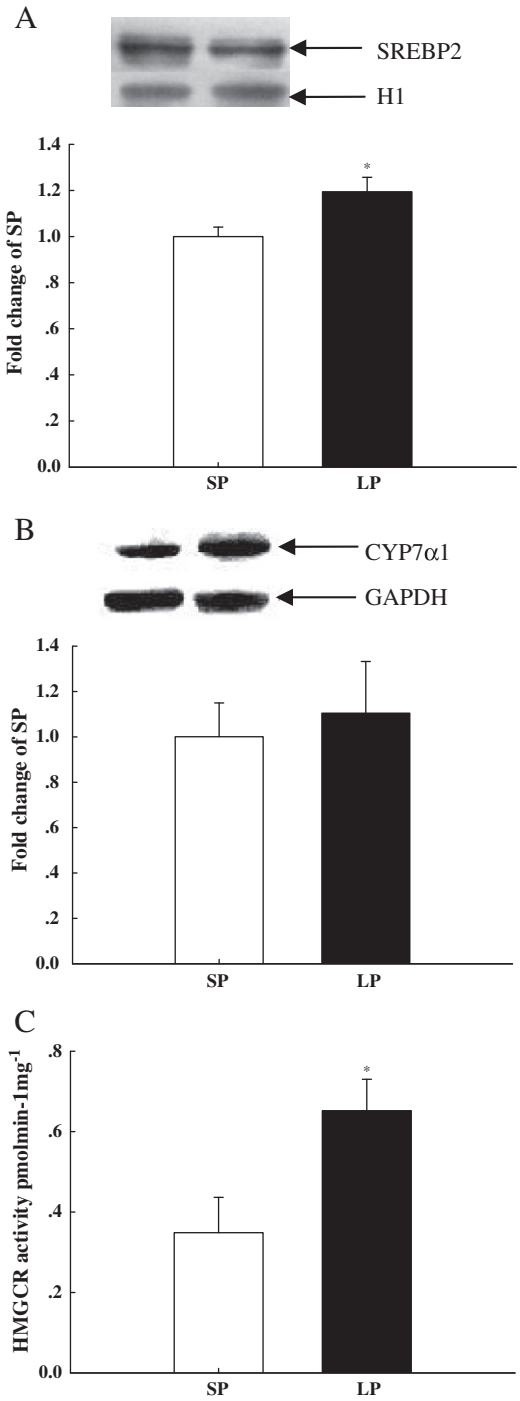

Fig. 2. Protein content and enzyme activity in the liver of weaning piglets. (A) SREBP2 protein; (B) CYP7α1 protein; (C) HMGCR activity. Values are means±S.E.M., n=7. *P<.05 compared with SP.

HDLC compared with the SP counterparts, no significant differences were observed for serum LDLC, HDLC or LDLC/HDLC ratio between the two groups. Moreover, hepatic expression of *LDLR* mRNA was not affected by maternal dietary protein level. These results implicate a decreased cholesterol pool rather than a disrupted cholesterol transport in LP piglets.

Cholesterol in the body originates from two sources: one is the diet if it contains animal-derived ingredients and the other is the de novo biosynthesis from acetyl-CoA. Sucking piglets obtain cholesterol from sow's milk. Cholesterol accounts for less than 3% of total lipids in

Table 5
MicroRNA expression in the liver of offspring at weaning piglets

Target genes	microRNA	SP	LP	*P* value
HMGCR	miR-23a	1.02±0.08	1.03±0.09	.919
	miR-23b	1.01±0.06	1.02±0.07	.964
	miR-29c	1.04±0.11	1.15±0.08	.428
	miR-497	1.03±0.11	0.98±0.13	.779
CYP7α1	miR-145	1.06±0.14	1.24±0.13	.372
	miR-181	1.06±0.15	1.00±0.05	.697
	miR-221	1.08±0.16	1.12±0.10	.831
	miR-499	1.09±0.16	1.13±0.24	.433
	miR-15b	1.23±0.09	1.07±0.33	.416

Values are means±S.E.M., n=7.

porcine milk, and milk cholesterol content is not affected by dietary protein level of the sow [39]. Therefore, the lower cholesterol in LP piglets may result primarily from decreased cholesterol biosynthesis.

In most mammalian species, the liver is a major site of cholesterol synthesis, although intestinal mucosa and some steroidogenic organs, such as adrenal cortex and gonads, also possess considerable cholesterol synthesis activity. In pigs, cholesterol synthesis occurs predominantly in the liver. Among the cerebrum, heart, ileum, kidney, liver, longissimus muscle, semitendinosus muscle and subcutaneous fat, the liver was the only tissue showing significant difference in cholesterol content between pigs selected for high or low serum cholesterol concentration, suggesting the importance of the liver in the regulation of cholesterol homeostasis in the pig [40].

Liver cholesterol content is determined by cholesterol transport, biosynthesis and transformation. In this study, maternal LP diet did not seem to change cholesterol transport; therefore, the lower liver cholesterol content in LP piglets is presumably due to reduced cholesterol biosynthesis and/or increased cholesterol transformation to bile acids. To our surprise, however, the decreased hepatic content of cholesterol in LP piglets was associated with significantly augmented hepatic expression of *SREBP2* and *HMGCR* mRNA, as well as increased SREBP2 protein in hepatic nuclear lysates and elevated HMGCR enzyme activity in liver cellular lysates. Since no significant changes were detected for hepatic mRNA expression of *LXR*, *FXR*, *LDLR*, *CYP27α1* or liver content of CYP7α1 protein, despite the up-regulated *CYP7α1* mRNA expression, we speculate that the de novo cholesterol biosynthesis mediated by SREBP2/HMGCR pathway is the main target affected by maternal dietary protein level in MS pigs. The activation of SREBP2 and HMGCR in the liver of LP piglets could be the result of feedback regulation of decreased liver cholesterol content, which agrees with the previous reports that lower liver cholesterol content increases the expression of SREBP2 and HMGCR [17,41].

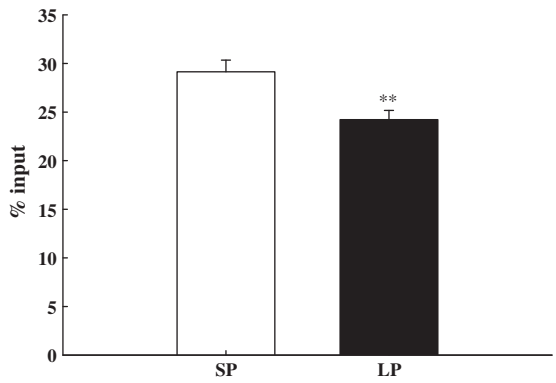

Fig. 3. DNA methylation at *HMGCR* promoter in the liver of weaning piglets. Values are means±S.E.M., n=7. **P<.01 compared with SP.

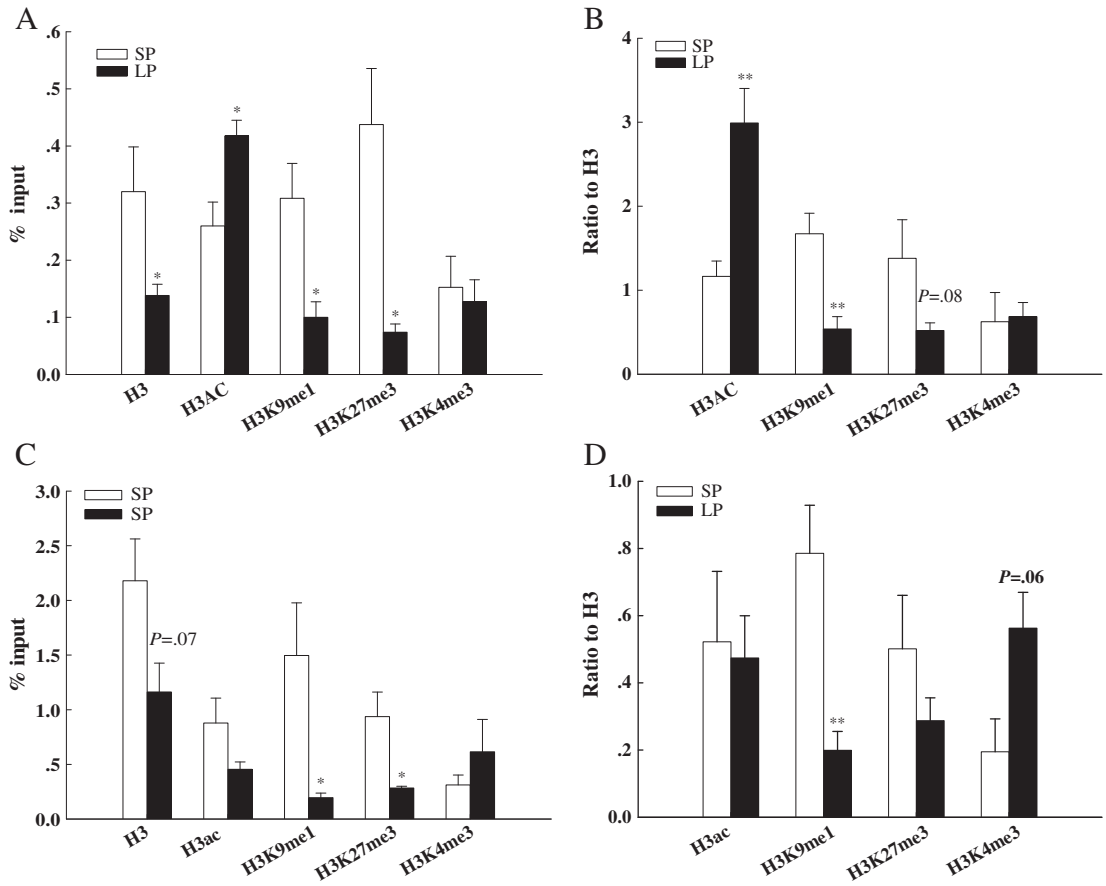

Fig. 4. Histone modification at *HMGCR* and *CYP7α1* promoter in the liver of weaning piglets. (A) Expressed as the percentage of the input; (B) expressed as the ratio relative to H3. Values are means±S.E.M., $n=7$. *$P<.05$ and **$P<.01$ compared with SP. Histone (C) expressed as the percentage of the input; (D) expressed as the ratio relative to H3. Values are means±S.E.M., $n=7$. *$P<.05$ and **$P<.01$ compared with SP.

A significant up-regulation of *CYP7α1* mRNA expression was observed in the liver of LP piglets, but liver content of CYP7α1 protein was not affected. The dissociation between *CYP7α1* mRNA expression and protein production implicates distinct regulatory mechanisms in *CYP7α1* transcription and translation. Cholesterol modulates its own catabolism via either positive or negative transcriptional regulation of *CYP7α1* gene, depending on the animal species [42]. Insulin was reported to repress *CYP7α1* transcription in cultured rat hepatocytes in an early publication [43]. Subsequent studies identified insulin responsive *cis*-acting elements on cholesterol 7alpha-hydroxylase gene promoter [44] and further clarified that insulin has dual effects on human *CYP7α1* gene transcription depending on the doses and durations of insulin treatment [45]. In this study, serum insulin concentration was significantly decreased in LP piglets (data not shown). However, it remains to be clarified whether the decreased cholesterol and/or insulin in LP piglets contribute to the up-regulation of hepatic *CYP7α1* mRNA expression.

The effect of maternal dietary protein on offspring cholesterol homeostasis is controversial. Plasma cholesterol level was reported to increase [4,5], decrease [3,6-8] or not change [2,9], and hepatic cholesterol content was shown to increase [4] or decrease [3] in offspring derived from dams fed LP diet. Numerous factors contribute to the divergent results, among which are animal species and the experimental design. The regulation of cholesterol metabolism seems to be highly species specific. Even within species, different breeds or lines respond to similar nutritional intervention differently [3,4]. To our knowledge, this is the first report describing the effect of maternal dietary protein level on offspring cholesterol metabolism in the pig. The dietary treatment for the sows was sustained throughout the period of gestation and lactation, and the data shown here represent the immediate effect of maternal LP diet. It is noted that the immediate and the long-term effects of maternal nutrition on offspring performance are often different or in some cases even opposite. For instance, maternal dietary protein induces opposite pattern of myosin heavy chain IIb [46] and myostatin [32] expression in LD muscle of MS offspring pigs at weaning and finishing stages. The long-term effects often represent the consequences of the compensatory mechanisms after the nutritional intervention ceases. For example, early dietary cholesterol causes minimal changes in cholesterol metabolism about 6 months after dietary exposure in human infants [47]. Similarly, we did not detect significant differences in either serum cholesterol concentration or hepatic cholesterol content between LP and SP pigs at finishing stage (8 months of age) when a complete compensation in body weight is achieved in LP piglets (data not shown). The activation of SREBP2/HMGCR pathway detected in LP piglets at weaning may contribute to the compensation of cholesterol biosynthesis in later life. However, in order to achieve a complete compensation, a sustained or prolonged activation of hepatic cholesterol biosynthesis is required during growth.

Epigenetic mechanism of gene regulation is considered "mitotically stable" [48] and may thus be involved in sustained or prolonged gene activation. Here we demonstrate, for the first time, the alterations in the epigenetic marks surrounding the promoters of *CYP7α1* and *HMGCR* in the liver of LP piglets. The genomic information of porcine SREBP2 gene is currently lacking, and we failed to clone the promoter region of this gene. Therefore, the

analysis of DNA methylation and histone modifications on SREBP2 gene promoter was excluded from this study. We chose *CYP7α1* and *HMGCR* for epigenetic analysis simply because their expression is significantly activated in LP piglets and the promoter sequences for these two genes are available for the pig. *HMGCR* gene activation is associated with the hypomethylation of the CpG-rich region in the promoter, together with increased H3 acetylation and decreased histone H3, H3K9me1 and H3K27me3. As no CpG islands are predicted in the *CYP7α1* promoter, the activation of *CYP7α1* transcription in LP piglets was accompanied by decreased H3, H3K9me1 and H3K27me3. Cytosine methylation in CpG islands located within gene promoters is generally associated with gene repression [49,50], while histone acetylation is usually associated with transcriptional activation [51]. Histone methylation exhibits either repression or activation effects on gene transcription, depending on the class of histones, the position of amino acid residues and the number of methyl groups (mono-, di- and trimethylation). H3K4me3 is generally regarded as an activation mark, while H3K9me1 and H3K27me3 are reported to induce transcriptional repression [52,53]. These notions are confirmed in the present study in which transcriptional activation of *HMGCR* and *CYP7α1* is associated with increase in activation marks, H3 acetylation for *HMGCR* and H3K4me3 for *CYP7α1*, and decrease in repressive marks, H3K9me1 and H3K27me3 for both *HMGCR* and *CYP7α1*. Histone H3 binding to the promoter of both *HMGCR* and *CYP7α1* is decreased, suggesting a relaxed chromatin structure around the promoter of activated genes. No differences were detected in miRNAs predicted to target 5′-UTR of *HMGCR* and *CYP7α1*genes, suggesting that miRNA-mediated posttranscriptional regulation may not be involved in the effect of maternal LP diet on hepatic *HMGCR* and *CYP7α1* expression in the pig.

Among all the epigenetic modifications, DNA methylation is considered to be the most stable, which may transmit the message obtained from early experience, e.g., maternal nutrition, to adult life. MeDIP analysis provides the overall methylation status of DNA fragments, usually CpG islands, while bisulfite sequencing or EpiTYPER-MassARRAY assay (Sequenom Inc.) allows quantitative analysis at each individual CpG site. The latter methods are more accurate and have higher resolution compared to MeDIP analysis; they are also more laborious and expensive. Moreover, there are also some limitations, such as incomplete conversion, degradation of DNA during bisulfite treatment and difficulties in primer design especially for GC-rich sequences. The quantitative analysis at each individual CpG site is best suited for investigating those CpG sites located within binding sequences of transcriptional factors. The present study was aimed to determine the overall methylation status of CpG island in *HMGCR* gene promoter; therefore, MeDIP-qPCR is adequate to serve the purpose. It remains a question for how long the *HMGCR* promoter maintains hypomethylated in LP piglets and whether the sustained activation of *HMGCR*, if it is the case, may eventually cause hypercholesteremia in LP pigs at a more advanced age than 8 months. Furthermore, it is of interest whether maternal LP diet may cause epigenetic abnormality in the germline to allow transgenerational inheritance of the phenotype in the deregulation of cholesterol homeostasis.

In conclusion, our results indicate that maternal LP diet during gestation and lactation affects hepatic cholesterol metabolism in weaning piglets through modifying the epigenetic regulation of *HMGCR* and *CYP7α1* genes, which implicates possible long-term consequences in cholesterol homeostasis later in adult life.

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