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Dietary protein restriction and excess of pregnant German Landrace sows induce changes in hepatic gene expression and promoter methylation of key metabolic genes in the offspring☆

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Abstract

Maternal nutrition during gestation has important effects on offspring gene expression mediated by DNA methylation. In order to evaluate the effect of restricted and excess protein intake during gestation, hepatic gene expression and DNA methylation of key metabolic genes *NR3C1*, *PPARα*, *HMGCR*, *PGC1α*, *INSR* and *CYP2C34* were investigated. Liver samples of German Landrace offspring were collected at Gestational Day 95, at birth, at weaning and from finisher pigs. Gene expression in foetal liver revealed significant differences between the control group (CO) and the low-protein group (LP) in *HMGCR* ($P<.0001$), *INSR* ($P=.0003$), *NR3C1* ($P=.020$) and *PGC1α* ($P=.003$). At birth *INSR* ($P=.032$), *PPARα* ($P=.0006$) and *CYP2C34* ($P<.0001$) showed significant differences between LP and CO. *CYP2C34* was significantly increased in the high-protein group (HP) compared to CO ($P=.001$). At weaning, *INSR* was significantly higher expressed in LP than in CO ($P=.018$). *HMGCR* showed a significant decrease of transcript amount in HP compared to CO ($P=.0006$). Furthermore, we studied the question whether gene expression differences between distinct diet groups are a result of differential DNA methylation status. CpG sites in the 5′-flanking region of *CYP2C34* showed a significant positive correlation with transcript amount in LP (nt −137: $R=0.67$, $P<.0001$; nt −112: $R=0.54$, $P=.003$). In *NR3C1* methylation, differences in the CpG island were negatively correlated with gene expression data in LP ($R=-0.34$, $P=.032$). The mean of methylation of *PPARα* over CpG sites from nt −220 to −11 was significantly increased in the LP group compared with CO ($P=.043$). These data suggest an influence of DNA methylation in nutrient-dependent transcriptional regulation of *NR3C1*, *PPARα* and *CYP2C34*.

Keywords: Protein restriction; Protein excess; Maternal diet; Foetal programming; Pig; Intrauterine growth restriction

1. Introduction

During the last decades, it became widely accepted that the prenatal period is a crucial phase of life within which foetal programming of gene expression patterns occurs causing lifelong consequences for the developmental outcome of the progeny [1]. Animal models of maternal protein restriction, especially using rodents, are well established for the study of nutrient–gene interactions as well as the development of growth restriction in offspring. Numerous studies using the maternal low-protein model give overwhelming evidence that intrauterine malnutrition can have long-lasting effects on gene expression of the progeny [2,3].

Among livestock species, pigs are most prevalently suffering from intrauterine growth retardation, which leads to enhanced piglet mortality and reduced muscle growth but increased fat deposition resulting in impaired meat quality traits with tremendous effects on farm animal production and welfare [4]. The underlying effects of foetal programming are still not completely understood. It is hypothesized that epigenetic effects manifesting during crucial pre- and perinatal developmental stages may play a key role in the relationship between intrauterine growth restriction and the susceptibility for metabolic disturbances in adulthood [5,6]. Especially, DNA methylation is a mechanism of epigenetic gene regulation that might be involved in the long-time programming of metabolism [7].

Appropriate candidate genes for investigations of the influence of maternal diet during gestation on foetal gene expression controlled by promoter-specific DNA methylation were chosen based on previous studies that give evidence for diet-dependent gene

Abbreviations: dpn, *dies post natum*; dpc, *dies post conceptionem*; LP, low-protein group; HP, high-protein group; CO, control group; nt, nucleotide.

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expression as well as their roles in the regulation of metabolism. Thus, the gene expression of glucocorticoid receptor gene (*NR3C1*) is tremendously affected by maternal malnutrition during gestation and lactation (*NR3C1*) [8–10] as well as of genes of the peroxisome proliferator-activated receptor family (e.g., *PPARα*) [9,11] that are involved in lipid homeostasis and carbohydrate metabolism [12–16]. Besides *NR3C1* and *PPARα*, further genes were investigated to be involved in key functions of the metabolic control belonging to fat metabolism (*HMGCR*, *PGC1α*), carbohydrate metabolism (*INSR*) and members of the cytochrome P450 superfamily (*CYP2C34*). In the liver, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, encoded by *HMGCR*, is the rate-limiting enzyme in cholesterol synthesis and catalyses the conversion of 3-hydroxy-3-methyl-glutaryl-CoA into mevalonic acid [17]. Long-term gene expression up-regulation is mediated by sterol regulatory element binding protein binding to the sterol regulatory element in the 5′ region of *HMGCR* [18]. Carbohydrate metabolism is also strongly influenced by gestational protein restriction. Studies in offspring of protein-restricted rat dams revealed an up-regulation of the hepatic insulin receptor gene (*INSR*) [19–21]. In the present study, we used a porcine model to analyse the effects of maternal protein restriction as well as excess during foetal development on expression of metabolic key gene and its DNA methylation profile on *NR3C1*, *PPARα*, *HMGCR*, *PGC1α*, *INSR* and *CYP2C34*.

2. Materials and methods

2.1. Animals, diets and sample collection

Animal care and tissue collection processes followed the guidelines of the German Law of Animal Protection, and the experimental protocol was approved by the Animal Care Committee of the State of Mecklenburg-Vorpommern (Germany). The animal experiment was performed as described previously [22]. In brief, primiparous German Landrace sows were inseminated at an age of 8 months. Pregnant gilts were randomly assigned to three diet groups throughout the gestation: control group (CO) containing 12.1% crude protein (CP, % of dry matter), high-protein group (HP) with 30% CP and low-protein group (LP) containing 6.5% CP. Diets were formulated to be isoenergetic (13.6 MJ ME/kg on average) [22]. In this study, liver samples of offspring derived from the sows of the three diet groups were collected and evaluated at Gestational Day 95 (n_{dpc95}=16 per diet group), at birth (dpn1; n_{dpn1}=10 per diet group), at weaning (dpn28; n_{dpn28}=16 per diet group) and at dpn188 (finisher pig; n_{dpn188}=16 per diet group).

At Gestational Day 95, foetuses were developed from six sows per diet group by caesarean section and foetal liver samples were immediately frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction. *Post natum* offspring was cross-fostered within 48 h after birth and nursed by foster sows fed control gestation (Provital RF R.324.0; Trede & von Pein, Dammfleth, Germany) and lactation (Provital LAC R.325.0; Trede & von Pein) diets meeting energy and nutrient recommendations. After weaning (28 dpn), offspring had *ad libitum* access to standard commercial diets (Trede & von Pein) formulated for post-weaning (dpn 29–48; Porcistart, 13.5% crude protein, 14 MJ ME/kg), growing (dpn 49–76: Porcibig, 12.1% crude protein, 13.8 MJ ME/kg; from dpn 77–105: Vormast Trafo TOP, 11.5% crude protein, 13.6 MJ ME/kg,) and finishing periods (dpn 106–188: Vital–Mast MM, 9.5% crude protein, 13 MJ ME/kg). Liver samples were collected at time point dpn1, dpn28 and dpn188. Samples were immediately frozen in liquid nitrogen and stored at −80°C. Husbandry conditions were already described in detail [22].

The body weights at Gestational Day 95 of the foetuses analysed here did not differ between the three diet groups. However, when scoring all neonates derived from the complete experiment, HP and LP offspring were growth restricted compared to CO offspring, but litter size did not differ [22]. At birth, LP piglets of the subset studied here were significantly lighter than piglets of the control group (CO 1.22±0.05 kg; LP 1.06±0.05 kg; P=.033); also, piglets of the HP group were lighter than piglets of the control group, but that difference was not significant. Birth weight differences did not persist until weaning at dpn28. Also, finisher pigs at dpn188 showed no significant differences in body weight depending on the maternal diet group.

2.2. DNA and RNA Isolation

Liver samples were powdered by mortar and pestle in liquid nitrogen. For DNA isolation, 50 mg of tissue was isolated by phenol–chloroform extraction using Eppendorf Phase Lock Gel-Tubes (PLG; heavy, 2 ml; Eppendorf, Germany) followed by precipitation and washing. Concentration and quality were checked by a spectrophotometer (ND1000; Nano Drop technologies, USA). DNA was stored at −20°C. RNA was isolated by Trizol extraction and cleaned with the RNeasy Mini Kit (QIAGEN, Germany) following the supplier's protocol. RNA was quantified spectrophotometrically and stored at −80°C.

2.3. Reverse transcription and quantitative real-time polymerase chain reaction (PCR)

First-strand cDNA was synthesized from 2 μg of total RNA with the SuperScript II Reverse Transcriptase Kit (Invitrogen, Germany) following the manufacturer's guide. Real-time quantitative PCR was performed using the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Germany). The amplification was conducted in duplicate according to supplier's instructions. Reactions were performed in a final volume of 10 μl using 5 μl of LightCycler 480 SYBR Green I Master (Roche), 2.0 μl of Aqua dest., 10 μM of each primer and 40 ng cDNA. The temperature profiles comprised an initial denaturation step at 95°C for 10 min and 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 10 s and extension at 72°C for 15 s. For all the assays, threshold cycles were converted to copy numbers using a standard curve generated by amplifying serial dilutions of an external PCR standard (10^7–10^2 copies). After completion of the amplification protocol, all samples were subjected to melting curve analyses and gel electrophoresis. Primers were obtained from Sigma-Aldrich. Primer sequences are given in Table 1. Expression levels were normalised to *RPL32* and *RPL10*.

2.4. Porcine promoter analysis and CpG island identification

Genomic sequences of porcine candidate genes were obtained from Ensembl Pig FPC map (http://www.ensembl.org/index.html; accessed January 2011). For multiple alignment of the 5′-upstream region, the genomic sequences of human, bovine, canine and murine sequences were retrieved from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/; accessed January 2011) and aligned using Mulan [23]. The 5′-flanking regions were checked for CpG islands and putative TFBS using EMBOSS CpGPlot [24] and MultiTF software tools [23]. We used the following criteria for CpG island prediction: minimum CpG island length ≥500 bp, CG content ≥55% and observed/expected ratio ≥0.6 [25].

2.5. Bisulfite direct sequencing of porcine CpG islands in 5′-flanking regions

Genomic DNA was treated with bisulfite by the EZ DNA Methylation Gold Kit (Zymo Research, USA) according to the supplier's instructions. In order to validate the efficiency of bisulfite conversion, non-methylated and 100% methylated DNA samples were generated, treated with bisulfite and used for bisulfite-specific PCR. For the generation of unmethylated samples (0%), genomic DNA was amplified using REPLI-g UltraFast Mini Kit (QIAGEN, Germany) and purified by phenol–chloroform extraction. To produce 100% methylated samples, genomic DNA was treated using the CpG methyltransferase kit (M.SssI 20,000 U/ml; New England Biolabs, Frankfurt/Main, Germany) and purified by phenol–chloroform extraction. The converted DNA was amplified by PCR. The primers used are given in Table 1. PCR was performed as follows using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Germany): hot start at 94°C for 4 min; 40 cycles with 94°C for 30 s, primer-specific annealing temperature for 40 s, 72°C for 1 min; followed by 72°C for 8 min. PCR for *PPARα* was performed as seminested PCR with the following temperature profile: First PCR: 94°C for 4 min; 40 cycles: 94°C for 30 s, 63°C for 40 s, 72°C for 1 min; 72°C for 8 min. Second PCR: 94°C for 4 min; 40 cycles: 94°C for 30 s, 58°C for 40 s, 72°C for 1 min; 72°C for 8 min. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN). The PCR products from bisulfite-modified DNA were directly sequenced by a commercial sequencing service (QIAGEN Sequencing Services).

All sequencing reaction mixes were based on the BigDye 3.1 Terminator chemistry (Applied Biosystems). Template amounts of 5 ng per 100 bases of fragment length and 10 pmol primer per reaction were used. Reactions were cycled in a GeneAmp PCR System 9700 (Applied Biosystems, German) and purified using DyeEx (QIAGEN). Data collection was carried out on a 3730xl DNA Analyzer (Applied Biosystems) equipped with 50-cm capillary arrays and POP 7 polymer. After data collection, the raw data channels from the generated result files were processed using custom-built software developed at QIAGEN.

Data processing involved the compensation of the different migration properties of the four dyes, baseline correction, peak detection and base calling. For each base, the area was calculated from the corresponding peak to measure the base's quantity. Bases that correspond to variable positions of CpG sites were identified by comparison of the obtained sequence with a reference sequence. The ratios of methylated vs. unmethylated species (C/T in forward reads, G/A in reverse reads) were reported. The values of the signal-to-background ratio (S/B) were used as quality control of quantification. S/B's of 20 and above were considered as high-quality samples and used for quantification of methylation status. The algorithm was developed and the procedure was previously validated [26,27].

Table 1
List of primer pairs used for sequencing, quantitative real-time PCR, pyrosequencing and direct bisulfite sequencing

Primer	5′–3′ Sequence	Amplicon (bp)	GenBank ID
Quantitative real-time PCR			
RPL32-RT-FP	AGCCCAAGATCGTCAAAAAG		
RPL32-RT-RP	TGTTGCTCCCATAACCAATG	165	NM_001001636.1
RPL10-RT-FP	CTGTGTTCGTCTTTTCTTCC		
RPL10-RT-RP	TCATCCACTTTTGCCTTCT	199	NM_001044543.1
CYP2C34-RT-FP	CTCTGGATCTCATGCACCAC		
CYP2C34-RT-RP	ACAGAGACAACGAGCACCAC	176	U35733.1
HMGCR-RT-FP	GTGCTGGTCTGTTTTGATTT		
HMGCR-RT-RP	TGCAGTGATTTGTTTTCTTG	159	NM_001122988.1
INSR-RT-FP	ATGCGATTGCCAGTGAAGT		
INSR-RT-RP	ATTCCGGGTTTCTCCAACA	131	XM_003123153.1
NR3C1-RT-FP	CAGTGATGGGAAAAGGAGAAAG		
NR3C1-RT-RP	TGTCCAACCGTGAAAAGTATG	158	NM_001008481.1
PGC1α-RT-FP	GTAAATCTGCGGGATGATGG		
PGC1α-RT-RP	TGGTGGAAGCAGGATCAAAG	208	AB106108.1
PPARα-RT-FP	CAGGTCACGCTGCTGAAGTA		
PPARα-RT-RP	AACTTGGGCTCCATGATGTC	164	NM_001044526
Pyrosequencing			
CYP2C34-PSQ-FP	TTTAGTTTTTGTTTTGAGGGGAG		
CYP2C34-PSQ-RP[Btn]	[Btn]TAAAACCCAAATACACACCCA	353	CU062549.3
CYP2C34-PSQ-SEQ1	GGAAGGATTGTTATTAAAGT		
CYP2C34-PSQ-SEQ2	GGAAGGATTGTTATTAAAGT		
INSR-PSQ-FP	GAAATAGTTTTAGGAGGGGAG		
INSR-PSQ-RP[Btn]	CACCCRCCAACCCAAATAC	258	NW_003534271.2
INSR-PSQ-SEQ1	GGAGGYGGTTTYGAT		
INSR-PSQ-SEQ2	TTTAGGGTTYGGAGTT		
PGC1α -PSQ-FP	ATTTTATTGAGGTAGAGGGTTG		
PGC1α -PSQ-RP[Btn]	[Btn] CAATCCATACAAAATCCTAATTACA	293	FP312716.2
PGC1α -PSQ-SEQ1	AGGGTTGTTTTTGAGT		
PGC1α -PSQ-SEQ2	TGGAGTTGAAATAGTTTGA		
Direct bisulfite sequencing PCR			
PPARα-BSP-FP1	TTAGTTTTTTTATTGGTGTTGTTAGGG		
PPARα-BSP-RP1	ACCTCCACTTCTACCCAACC		
PPARα-BSP-FP2	GGTAGGAGGTGGGAGTYGTTAG		
PPARα-BSP-RP2	CCTAACACCTAAAACTACRATCC	371	FP016092.1
NR3C1-BSP-FP1	AGTTYGTAAAATGGAGGAGGAG		
NR3C1-BSP-RP1	ACCRCCCCTACAATTACC	247	CU928713.21
NR3C1-BSP-FP2	GAGGGAATYGAGTTTTTTTAGT		
NR3C1-BSP-RP2	CRTATCTAACCTTCCAATCC	306	CU928713.21
HMGCR-BSP-FP1	TAGGTTAGGTTTTGGGTTGTAG		
HMGCR -BSP-RP1	TCTAACCAATCATCTAAAAATTACTC	268	CU694556.2
Sequencing			
PPARα-FP	GCCTCTCCCTCCTCAGTTTC		
PPARα-RP	CGACCACGCTGAAGGAAG	771	FP016092.1

RT, Real-time PCR; PSQ, pyrosequencing; BSP, bisulfit sequencing PCR; FP, forward primer; RP, reverse primer; SEQ, sequencing primer.

2.6. Bisulfite pyrosequencing of porcine CpG islands in 5′-flanking regions

Determination of the methylation status of single CpG sites within the 5′-flanking region of *INSR*, *CYP2C34* and *PGC1α* was performed by pyrosequencing following the protocol of Tost and Gut [28] using the PSQ system (Biotage, Uppsala, Sweden). Bisulfite-converted DNA was subjected to PCR amplification. The PCR condition was a hot start at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, primer-specific annealing temperature for 40 s and 72°C for 30 s, followed by 72°C for 8 min. Used primers are given in Table 1. The biotinylated PCR product was used for pyrosequencing reaction following the supplier's protocols. Thus 20 μl of the PCR product was immobilized to Streptavidin Sepharose beads (GE Healthcare, Piscataway, NJ, USA) followed by annealing to sequencing primer (5 μM) for 2 min at 80°C. Pyrosequencing reactions were performed in the PSQ 96MA Pyrosequencer (Biotage). Data recording was performed with the PSQ 96MA software (Biotage). The percentage of C was calculated relative to the sum of the amounts of C and T at each CpG site. Complete conversion of cytosine at a non-CpG site ensured successful bisulfite conversion.

2.7. Statistical analyses

Statistical analyses were performed with the SAS 9.2 software package for Windows (SAS Institute, Cary, NC, USA). Real-time data were normalised as described previously [29]. Differences between diet groups at time points dpc95, dpn1, dpn28 and dpn188 for body weight, methylation status as well as real-time PCR data were analysed by the general linear model (GLM) procedure using the method of least squares to fit GLMs and a post hoc Tukey–Kramer multiple comparison procedure. Dietary group (CO, HP, LP), class of birth weight (lightweight, heavy), sex (female, male) and sow nested within a diet group were considered as fixed effects. Weight at slaughter was included as covariable. Statistical dependence between methylation status and real-time PCR data was estimated using Spearman's rank correlation coefficient (designated as *R*). Values are given as least-squares means±S.E. $P<.05$ was considered to be statistically significant.

3. Results

3.1. CpG island characterisation and sequence analysis

Analysis of porcine *NR3C1*, *HMGCR*, *INSR* and *PPARα* revealed CpG islands within their 5′-flanking region (Fig. 1). *NR3C1*, *HMGCR* and *PPARα* showed large CpG islands of approximately 1 kb (Table 2). The schematic structure of the CpG island position relative to exon 1 and the 5′-flanking region is shown in Fig. 1. *PGC1α* and *CYP2C34* do not possess CpG islands in their 5′-flanking region. There are three CpG

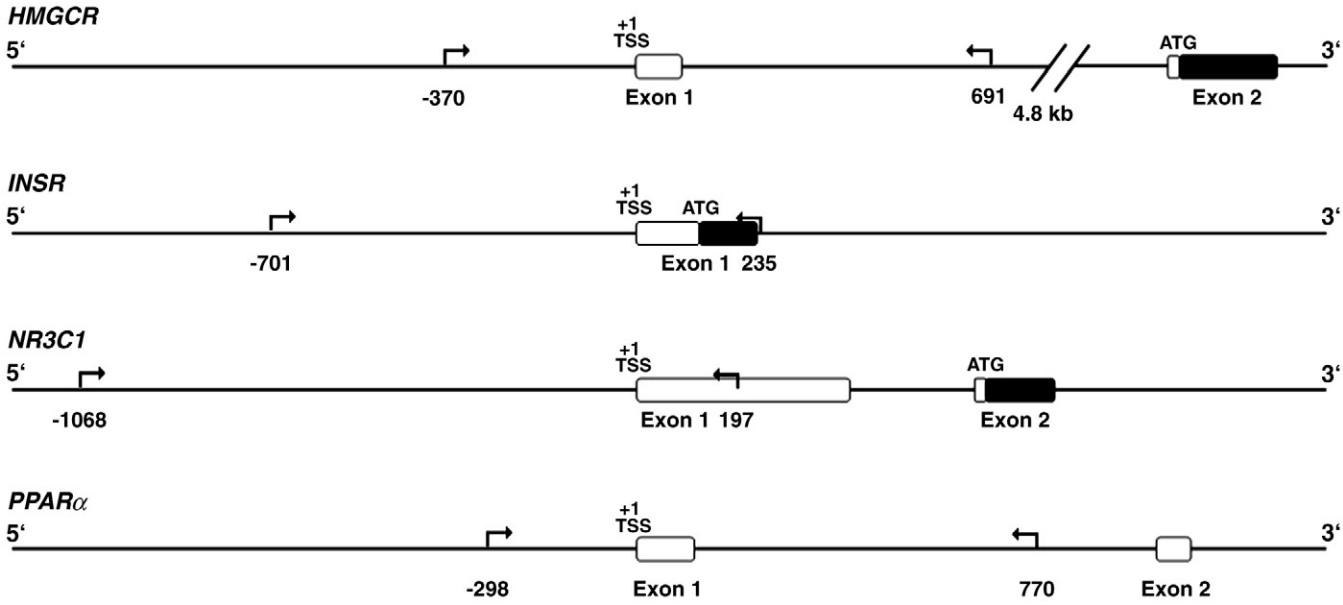

Fig. 1. Structure of CpG island within porcine candidate genes; schematic presentation of the CpG island and the exon 1 of *HMGCR*, *INSR*, *NR3C1* and *PPARα*; arrows with numbers indicate the start and end position of a CpG island relative to TSS; white boxes, untranslated exon; black boxes, coding sequence; TSS, transcription start site; ATG, start codon.

sites in the region from nt −1000 to transcription start site of *CYP2C34* at positions −349, −137 and −112. *PGC1α* shows 14 CpG sites within 1000 bp upstream of the ATG start codon at nt −955, −791, −637, −533, −396, −384, −374, −343, −291, −256, −219, −214, −21 and −17.

3.2. Gene expression analyses

The results of quantitative gene expression analyses in liver tissue are summarised in Table 3. At dpc 95, foetuses of the LP group showed a significantly higher transcript amount of *NR3C1* compared to those of the CO and HP groups, respectively (P=.020; P=.029, respectively). This difference was no longer obvious at dpn1 and afterwards. *PPARα* gene expression at Gestational Day 95 did not differ in the HP and LP groups compared to the control group. At dpn1, *PPARα* expression was significantly decreased in LP as compared to CO (P=.0006) and HP groups (P=.004). At dpn28 and dpn188, there were no longer differences in *PPARα* gene expression among diet groups. *PGC1α* revealed a significantly higher transcript amount in the LP than in the CO group at dpc95 (P=.003). At dpn1, the *PGC1α* transcript amount of HP animals showed a significant increase of gene expression in comparison to CO (P=.046). Maternal protein restriction resulted in significant differences in the expression of *HMGCR* at dpc95 and dpn28. At foetal stage, the *HMGCR* transcript amount of CO was significantly higher than that of LP (P<.0001). At dpn1, this difference is still present as a trend (P=.087). At dpn28, the HP group had a significantly decreased transcript amount in comparison to CO (P=.0006). At dpc95, dpn1 and dpn28, *INSR* showed a significantly increased transcript amount in LP animals compared to CO (P=.0003, P=.032, P=.018, respectively). *CYP2C34* gene expression data revealed significant differences between the offspring of the three maternal diet groups at dpn1. HP as well as LP piglets showed significantly increased transcript amounts in comparison to CO (P=.001; P<.0001, respectively). At foetal age, at dpn28 and at dpn188, no differences were obvious.

Table 2
CpG island report; given are the CpG island characteristics of the candidate genes (EMBOSS CpGPlot/CpGReport; settings: observed/expected ratio 0.60, %C+%G >50.00, length >500 bp)

Gene	CpG Island length (bp)	CpG Island position relative to TSS		Number of CpG sites within an island	Percent CG	Observed/ expected ratio
		Start	End			
HMGCR	1061	−370	691	91	63.90	0.85
INSR	936	−701	235	119	76.50	0.86
NR3C1	1265	−1068	197	172	75.73	0.96
PPARα	1068	−298	770	128	75.66	0.84

3.3. Effect of maternal dietary protein level during gestation on the methylation of INSR, CYP2C34 and PGC1α CpG sites in the 5′-flanking region based on pyrosequencing

Gene expression of *CYP2C34* in newborn piglets (dpn1) showed significant differences between CO, HP and LP. Thus, we isolated DNA from newborn piglets (dpn1) for methylation analysis. Pyrosequencing results of CpG sites −349, −137 and −112 in the *CYP2C34* 5′-flanking region at dpn1 are given in Fig. 2A. The CpG sites are highly methylated, and positions −137 and −112 revealed significant differences between CO and LP animals. Additionally, methylation of both CpG sites showed a significant positive correlation with transcript amount (nt −137: R=0.67, P<.0001; nt −112: R=0.54, P=.003).

Pyrosequencing was also performed for CpG sites −219, −214, −21, −17 and +5 in the *PGC1α* 5′-flanking region at Gestational Day 95. CpG at nt −219 was completely unmethylated, and CpG at nt −214 revealed slight methylation up to 5%. Methylation at positions −21, −17 and +5 reached levels between 10% and 20%. None of the CpG sites showed significant differences between piglets of the three maternal diet groups (Fig. 2B). However, CpG at position −21 showed a tendency to increased methylation in LP compared to CO piglets (P=.075).

For *INSR*, the methylation pattern of 15 CpG sites within a 258-bp-long fragment ranging from nt −438 to nt −181 in the 5′-flanking

Table 3
Transcript amounts in liver at dpc95 ($n=48$, respectively), dpn1 [$n=48$, respectively; but *PGC1α*, *PPARα*, *CYP2C34* ($n=30$, respectively)], dpn28 ($n=48$, respectively) and dpn188 ($n=48$, respectively)

Age	Gene	Maternal diet (least-squares means±S.E.)						Statistical comparison (*P* value)		
		CO		HP		LP		CO vs. HP	CO vs. LP	HP vs. LP
dpc95	*CYP2C34*	2.13E+05	±7.77E+04	2.74E+05	±8.27E+04	2.77E+05	±7.77E+04	–	–	–
	HMGCR	1.17E+06	±4.91E+04	1.01E+06	±5.19E+04	7.63E+05	±4.89E+04	0.094	<0.0001	0.006
	INSR	9.03E+02	±1.97E+02	1.12E+03	±2.08E+02	2.27E+03	±1.96E+02	–	0.0003	0.002
	NR3C1	8.73E+04	±4.48E+03	8.80E+04	±4.73E+03	1.06E+05	±4.45E+03	–	0.020	0.029
	PGC1α	3.63E+04	±1.70E+03	3.65E+04	±1.79E+03	4.55E+04	±1.69E+03	–	0.003	0.005
	PPARα	3.29E+05	±1.72E+04	3.07E+05	±1.82E+04	3.02E+05	±1.72E+04	–	–	–
dpn1	*CYP2C34*	7.11E+04	±5.49E+04	4.63E+05	±5.80E+04	5.89E+05	±5.49E+04	0.001	<0.0001	–
	HMGCR	3.61E+05	±5.34E+04	2.66E+05	±5.64E+04	1.98E+05	±4.92E+04	–	0.087	–
	INSR	1.04E+03	±1.57E+02	1.63E+03	±1.94E+02	1.74E+03	±1.71E+02	0.094	0.032	–
	NR3C1	5.59E+04	±7.71E+03	4.78E+04	±8.16E+03	5.23E+04	±7.11E+03	–	–	–
	PGC1α	7.80E+04	±1.53E+04	1.40E+05	±1.62E+04	9.56E+04	±1.53E+04	0.046	–	–
	PPARα	1.28E+05	±5.85E+03	1.18E+05	±5.70E+03	8.38E+04	±5.74E+03	–	0.0006	0.004
dpn28	*CYP2C34*	8.21E+06	±1.00E+06	6.54E+06	±1.00E+06	7.39E+06	±1.00E+06	–	–	–
	HMGCR	2.84E+05	±2.56E+04	1.20E+05	±2.56E+04	2.87E+05	±2.55E+04	0.0006	–	0.0005
	INSR	3.63E+02	±4.29E+01	4.33E+02	±3.74E+01	5.38E+02	±3.53E+01	–	0.018	–
	NR3C1	3.39E+05	±3.35E+04	2.85E+05	±3.35E+04	3.80E+05	±3.34E+04	–	–	–
	PGC1α	3.58E+04	±5.11E+03	4.16E+04	±5.08E+03	4.46E+04	±5.10E+03	–	–	–
	PPARα	3.70E+05	±2.38E+04	3.74E+05	±2.36E+04	3.93E+05	±2.38E+04	–	–	–
dpn188	*CYP2C34*	5.74E+05	±1.30E+05	8.83E+05	±1.23E+05	9.68E+05	±1.38E+05	–	–	–
	HMGCR	6.60E+04	±9.75E+03	7.01E+04	±1.00E+04	4.45E+04	±1.06E+04	–	–	–
	INSR	1.77E+03	±3.42E+02	1.84E+03	±3.16E+02	2.37E+03	±3.23E+02	–	–	–
	NR3C1	4.16E+04	±4.19E+03	4.88E+04	±4.31E+03	3.93E+04	±4.55E+03	–	–	–
	PGC1α	3.13E+04	±4.88E+03	3.79E+04	±4.62E+03	2.62E+04	±5.18E+03	–	–	–
	PPARα	4.72E+05	±5.68E+04	4.83E+05	±5.38E+04	4.89E+05	±6.02E+04	–	–	–

region was determined at dpc95. The CpG sites at these positions were unmethylated (methylation level 0%) in each diet group.

3.4. Effect of maternal gestational protein restriction on the methylation profile of NR3C1, PPARα and HMGCR promoter based on direct sequencing

Hepatic DNA from the foetuses at Gestational Day 95 was used for methylation analysis of the *NR3C1* promoter. A CpG plot of a DNA region ranging from nt −2000 to +103,800 comprising the whole porcine *NR3C1* sequence showed a 1265-bp-long CpG island from nt −1068 to +197 (Fig. 3). The schematic structure of the *NR3C1* gene and the position of the CpG island are given in Figs. 1 and 3. DNA methylation of individual CpG sites within the *NR3C1* promoter is given in Fig. 5A. Nine CpG sites at nt −645, −640, −545, −535, −505, −487, −481, −471 and −468 showed significant differences between the CO and LP groups ($P<.050$). Between CO and HP, three CpG sites at positions −645, −505 and −468 differed significantly

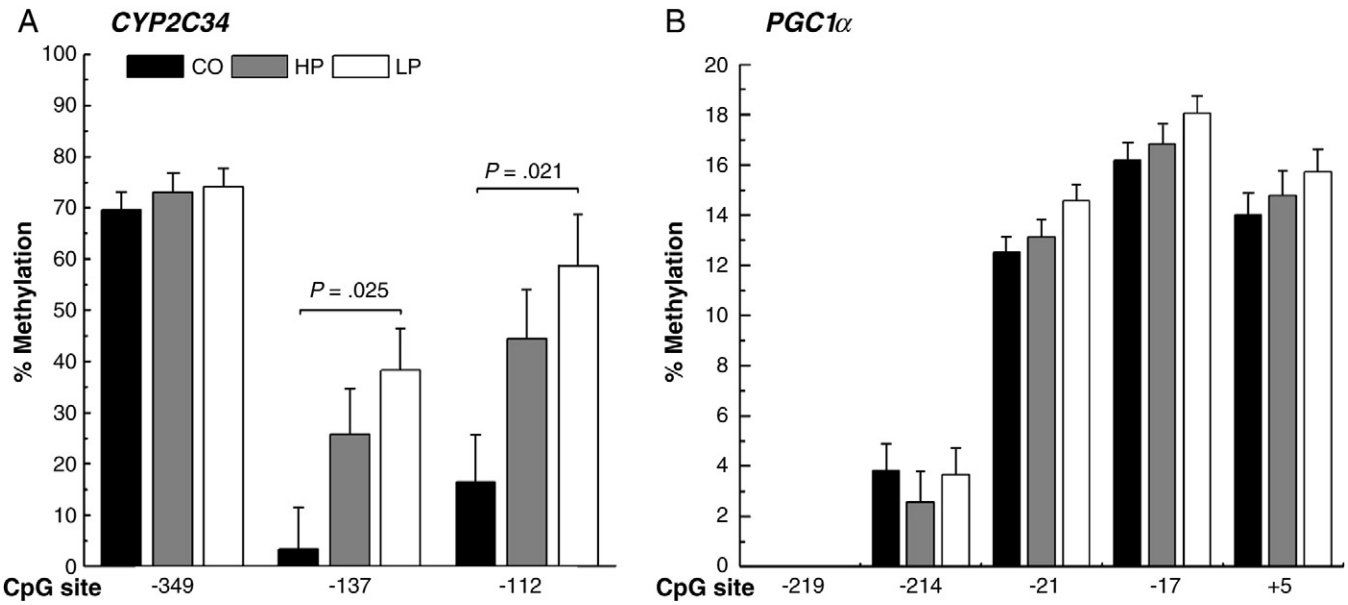

Fig. 2. Pyrosequencing results. (A) *CYP2C34*: Methylation of three individual CpG sites within the *CYP2C34* 5′-flanking region in the liver tissue of pigs from three diet groups at dpn1 ($n=30$). (B) *PGC1α*: Methylation of five individual CpG sites within the 5′-flanking region in the liver tissue of pigs from three diet groups at dpc95 ($n=48$). Given are least-squares means±S.E.

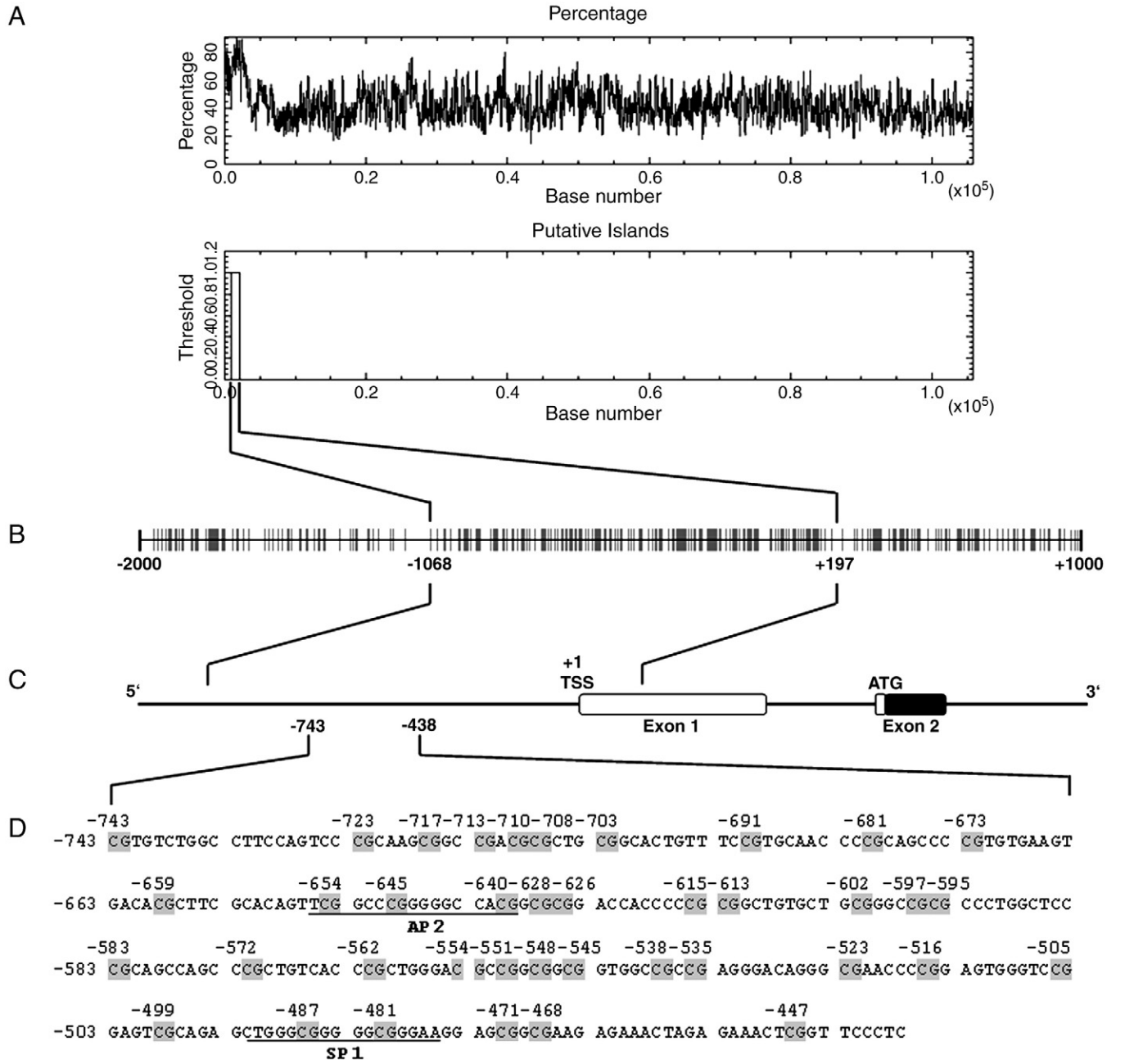

Fig. 3. (A) CpG Island identification in the porcine *NR3C1* gene using EMBOSS CpGPlot (settings: observed/expected ratio >0.60, %C+%G >60.00, length >500 bp). (B) CpG Density report: CpG sites are indicated by grey bars. (C) Structure of the porcine *NR3C1* gene: white boxes, 5′ UTR; black boxes, coding sequence; TSS, transcription start site. (D) Nucleotide sequence of the investigated part of the CpG island showing individual CpG sites and putative transcription factor binding sites (MultiTF): SP1, specific factor 1/GC-Box-factor; AP2, activator protein 2.

(P<.050). Absolute methylation level revealed values up to 20%. The average methylation over CpG sites from nt −645 to −468 was significantly decreased by about 20% in HP (P=.008) and LP (P=.006) compared to CO, respectively. Mean level of methylation of CpG sites differing between LP and CO showed a significant negative relationship (R=−0.34) between methylation and transcript abundance at P=.032.

Within the 5′-flanking region of *PPARα* ranging from nt −220 to the transcription start site, 33 CpG sites were investigated at dpn1. A CpG plot of the *PPARα* gene region showed a 1068-bp CpG island from nt −298 to +770 (Fig. 4). The schematic structure of the *PPARα* gene and the position of the CpG island are given in Figs. 1 and 4. Methylation levels of individual CpG sites ranged from about 1% to 22% (Fig. 5B). The HP group presented significantly decreased methylation at CpG positions nt −127 and −65. In LP animals, two sites at nt −65 and −27 showed significantly different methylation in comparison to CO (P<.050). In addition, for CpG sites at nt −211, −151, −128, −99, −60 and −39, a tendency to increased methylation in LP compared to CO was obvious due to the high interindividual variability of the methylation levels (Fig. 5B). However, the mean methylation over CpG sites from nt −220 to −11 was significantly increased in the LP group compared to the CO group (P=.043).

The methylation pattern of *HMGCR* was determined within a 268-bp-long fragment ranging from nt −338 to nt −71 and containing 17 CpG sites at dpc95 and dpn 28. The CpG sites in this

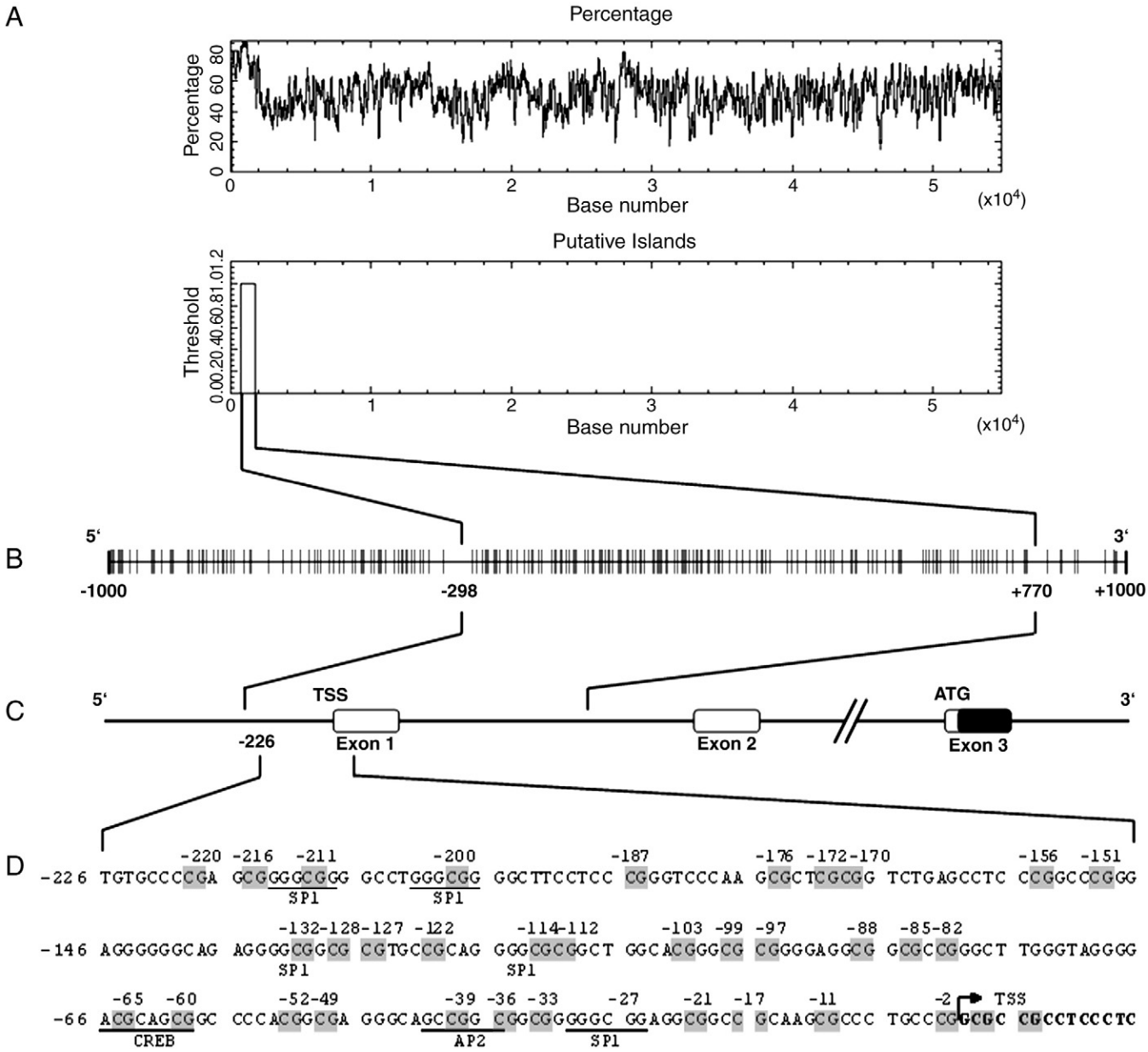

Fig. 4. (A) CpG Island identification in the porcine *PPARα* gene using EMBOSS CpGPlot (settings: observed/expected ratio >0.60, %C+%G >60.00, length >500 bp). (B) CpG Density report: CpG sites are indicated by grey bars. (C) Structure of the porcine *PPARα* gene: white boxes, 5′ UTR; black boxes, coding sequence; TSS, transcription start site. (D) Nucleotide sequence of the investigated part of the CpG island showing individual CpG sites and putative transcription factor binding sites (MultiTF): SP1, specific factor 1/GC-Box-factor; AP2, activator protein 2.

region were hypomethylated in each diet group (average methylation level <1%).

4. Discussion

Maternal protein restriction as well as excess during gestation affects the phenotypic outcome of offspring. Thus, in the present study, offspring gene expression of key metabolic genes was investigated in the liver as a major tissue of metabolic regulation being susceptible to maternal nutrient intake during gestation. Additionally, the methylation status of CpG sites within the 5′-flanking region of candidate genes was examined to study nutrient-induced promoter-specific methylation in a porcine model of differing gestational dietary protein intake.

NR3C1 gene expression was significantly enhanced in the liver of foetuses (dpc95) from protein-restricted mothers (LP). This was also observed in studies using the rodent low-protein model [8,9,30]. This is attributed to increased glucocorticoid hormone activity in maternally protein-restricted offspring [31–33]. Furthermore, the glucocorticoid receptor is indispensable for appropriate intrauterine development. Thus, maternal diet-related changes in *NR3C1* gene expression may have consequences for the transcriptional regulation of target genes and may lead to life-long phenotypic alterations [33]. Additionally, increased glucocorticoid receptor gene expression is linked to increased glucocorticoid hormone exposure in the offspring of protein-restricted mothers [8,32]. It is supposed that enhanced glucocorticoid level in rat foetuses of protein-restricted mothers has its source in the maternal circulation and crosses the placenta to influence the foetal

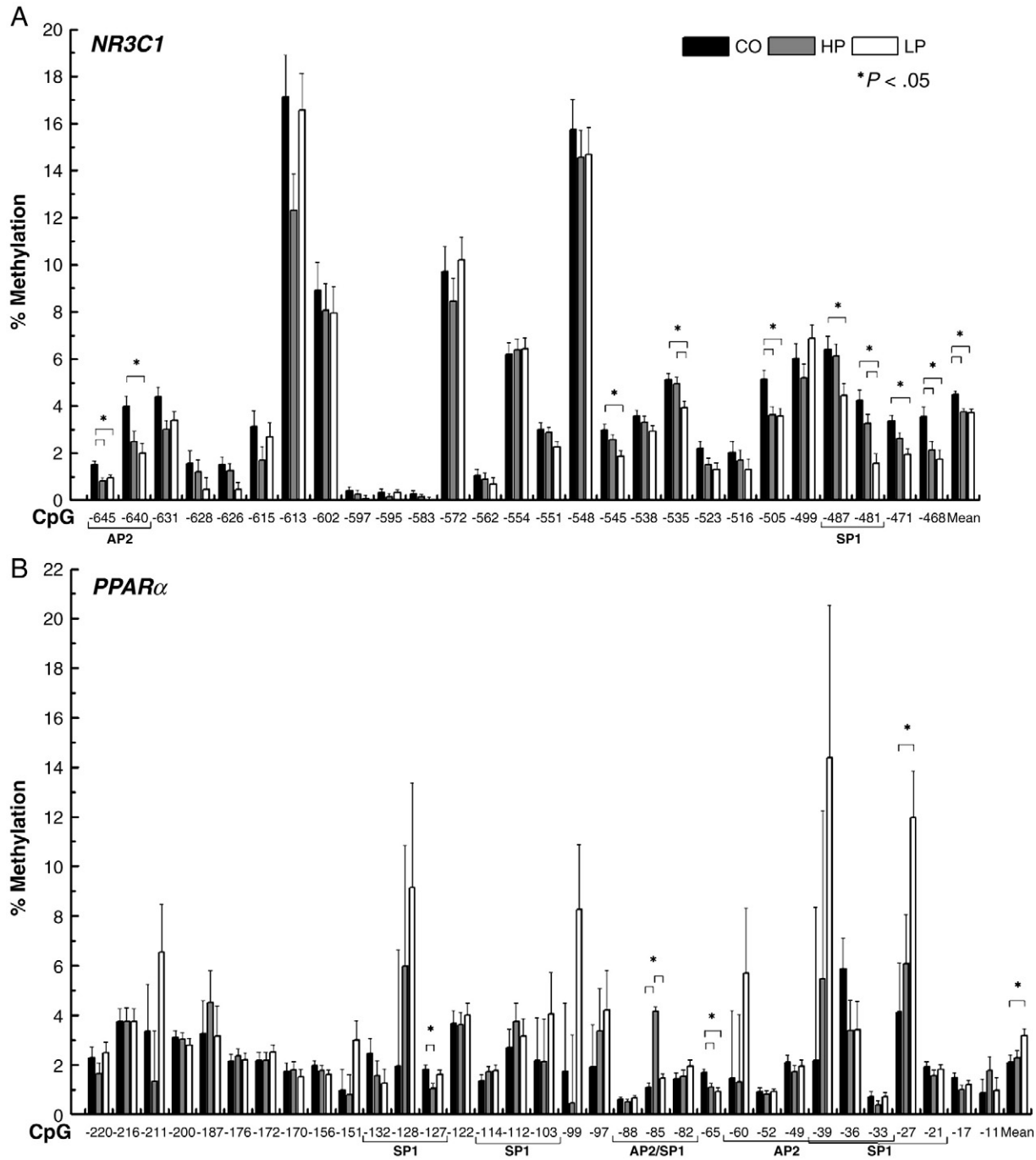

Fig. 5. (A) Bisulfite sequencing results of *NR3C1*. Methylation of individual CpG sites in the *NR3C1* promoter region in the liver tissue of pigs from three diet groups at dpc95 ($n=48$). (B) Bisulfite sequencing results of *PPARα*. Methylation of individual CpG sites in the *PPARα* promoter region in the liver tissue of pigs from three diet groups at dpn1 ($n=60$). SP1, Specific factor 1/GC-Box-factor; AP2, activator protein 2. *$P<.050$. Given are least-squares means±S.E.

hypothalamic pituitary adrenal (HPA) axis [34,35]. Intrauterine programming of HPA axis may be a link between the occurrence of low birth weight due to gestational malnutrition and the prevalence of adult metabolic diseases [36,37]. In pigs, a relation between low birth weight and increase in HPA axis function in later life is evident, suggesting early life programming with an impact on physiology and metabolism [38].

The influence of maternal protein malnutrition during gestation on lipid metabolism in adipose tissue has been widely investigated [39–42], but there is less information about changes in hepatic fat metabolism [43]. Hence, in the present study, we investigated the gene expression of a key regulator of fat metabolism, *PPARα*, and its co-regulator, *PGC1α*, as well as *HMGCR* in porcine liver. The transcript amounts of *PGC1α* and *HMGCR* showed differences in foetal liver tissue and of *PPARα* in the liver from newborn piglets, indicating an impact of maternal protein restriction on fat metabolism in offspring liver during early development. Interestingly, in rat offspring exposed to a maternal low-protein diet during pregnancy, hepatic steatosis was reported [43]. However, experimental data dealing with maternal protein restriction during gestation on fat metabolism in

offspring revealed inconsistent results due to different animal models, (postnatal) feeding regimes and/or offspring ages at measurement [39–41,44,45].

Both *PPARα* and *PGC1α* are engaged in the transcriptional up-regulation of genes involved in fatty acid metabolism and transport as well as peroxisome proliferation [46,47]. *PPARα* gene expression was less in LP compared to CO at dpn1 but showed no differences at weaning age (dpn28) and at dpn188 in the present study. This result is consistent with an experimental study in Wistar rats reporting no differences in hepatic *PPARα* mRNA expression in 4-week-old offspring (weaned at 4 weeks of age) of mothers fed control or low-protein diet throughout gestation [43]. In contrast, another study in Wistar rats reported significantly higher *PPARα* mRNA expression in the liver of 34-day-old offspring from protein-restricted mothers in comparison to the control group [9]. Nevertheless, significant differences in the gene expression of metabolic key genes *PPARα*, *PGC1α* and *HMGCR* in foetal and newborn pigs demonstrate the effects of gestational protein restriction as well as protein excess on fat metabolism in offspring [48].

It is noteworthy here that significant differences in the gene expression between HP and CO occurred at dpn1 in *PGC1α* ($P=.046$) and at dpn28 in *HMGCR* ($P=.0006$), suggesting the effects of gestational protein excess on offspring fat metabolism. That high-protein diets might influence hepatic lipid metabolism in the offspring was previously shown in rodents [49,50].

Insulin is a regulator not only of glucose but also of lipid and protein metabolism in the liver, adipose and muscle tissue acting via its insulin receptor [51]. The insulin receptor, a protein tyrosine kinase, becomes autophosphorylated after insulin binding and thereafter activates the insulin signalling pathway by further phosphorylation of intracellular downstream target proteins [52]. *INSR* content can be regulated either transcriptionally by glucocorticoids and nutritional supply [53–56] or post-transcriptionally by insulin [55,57]. Glucocorticoids directly stimulate *INSR* transcription, while insulin down-regulates *INSR* amount by degradation [55]. In the present study, there was a significant increase in *INSR* gene expression in LP compared to CO at dpc95, dpn1 and dpn28, suggesting increased insulin sensitivity during foetal as well as early postnatal development. These findings are supported by studies in offspring of protein-restricted rat dams revealing an up-regulation of hepatic insulin receptors in adipocytes and muscle tissues [19–21]. Thus, there may be increased *INSR* transcription due to increased glucocorticoid hormone exposure in the offspring of protein-restricted mothers which is also reflected by the increased *NR3C1* gene expression data of the recent study.

Cytochrome P450 superfamily member *CYP2C34* revealed significantly increased transcript amounts in LP as well as in HP compared to CO in newborn piglets (dpn1). Information about the influence of maternal diet on members of the P450 enzymes is scarce, and the function of *CYP2C34* is still unknown [58]. However, a study using Sprague-Dawley rats fed a perinatal low-protein diet revealed no effect of the maternal diet on the activity and protein expression of *CYP2D1* and *CYP2E1* [59]. In contrast, liver CYP-related enzyme activity and protein content of the CYP1A, CYP2B and CYP2E subfamilies were decreased in weanling rats fed a protein-restricted diet for 45 days [60]. Moreover, feeding mice a high-protein diet during gestation and lactation revealed the effects on hepatic offspring genes related to xenobiotic metabolism [61]. Thus, maternal dietary protein supply affects cytochrome P450 complex members and suggests an influence on xenobiotic metabolism with altered sensitivity to the toxic and carcinogenic effects of chemicals [62–64].

The strong impact of maternal low-protein diet on foetal (dpc95) and newborn (dpn1) offspring gene expression may reflect the malnutrition of LP foetuses during pre- and perinatal development. Although protein accretion in foetal pig is low in early and mid gestation, it increases dramatically in late gestation caused by rapid growth and weight gain with ~18.5-fold increased requirement for amino acids [65,66]. Thus, during the last quarter of gestation, foetuses are exceedingly prone to maternal protein restriction developing metabolic adaptations to their nutritional situation. However, our data do not show a long-term impact on gene expression due to maternal protein malnutrition during gestation. This may be due to the fact that offspring was cross-fostered shortly after birth and reared by adequately fed foster sows. Animal studies revealed a poor milk yield in gestational protein-restricted females [67–69]. Thus, the prenatal phase may be crucial for the foetal programming of certain gene expression pattern in the offspring liver, but also the pre-weaning period may have a major impact on the offspring [12,13,70]. In contrast, the influence of maternal high-protein diet on offspring gene expression of key metabolic genes in the offspring might be explained by a catabolic situation in the maternal organism caused by the need of excess nitrogen disposal and the lack of glucose leading to a depletion of maternal body fat [22]. Amino acid catabolism including urea synthesis, ketogenesis and gluconeogenesis is increased by high-protein diet [71,72].

To gain insight into the mechanisms of how maternal nutrition affects metabolism and expression of genes in the liver of the offspring, the methylation level of CpG sites within the 5′-flanking region of *HMGCR*, *INSR*, *NR3C1*, *CYP2C34*, *PPARα* and *PGC1α* was examined.

Within the CpG island of *NR3C1* and *PPARα*, significant differences in methylation levels were obvious. In both genes, the observed methylation level was quite low and varied between 2% and 20%. Nevertheless, there were significant differences in the methylation of individual CpG sites of LP and HP in comparison to CO offspring. Moreover, there was a significant negative correlation between CpG methylation level and gene expression, thus suggesting an involvement of promoter CpG island methylation in the transcriptional regulation of *NR3C1* and *PPARα*. Interestingly, in several studies, the methylation level of differentially expressed genes was also quite low (below 20%) [11,73,74], indicating a sensitive transcriptional control via CpG methylation. This might be due to the indispensability of a precise transcriptional control in phenotype formation during prenatal environment in contrast to gene imprinting with the result of complete gene silencing [11].

Several significantly methylated CpG sites within the 5′-flanking region of porcine *NR3C1* coincide with putative transcription factor binding sites, implying a functional significance of this region. The location of differently methylated CpG dinucleotides within putative transcription factor binding sites indicates a potential regulation of *NR3C1* transcription by DNA methylation. In humans and rodents, multiple alternative first exons of the *NR3C1* gene were discovered [75–78]. The porcine first exon and its 5′-flanking promoter region investigated here correspond with human and murine exon 1C and associated promoter, respectively [76–78]. The human promoter was already characterised as a TATA-less but GC-rich promoter containing multiple GC box elements [79]. The predicted specific protein 1 (SP1) binding site that overlaps with the CpG sites at nt −487 and −481 in the porcine promoter corresponds with a previously experimentally identified SP1 binding site in the human orthologue [80,81]. This binding site is highly conserved between human, mouse, cow and pig sequences. In addition, we observed two significantly different methylated CpG sites within a putative AP2 site conserved between human, mouse, cow and pig sites in the porcine sequences (LP nt −645, −640; Fig. 5A). Inhibition of binding of transcription factors SP1 and AP2 by methylation within and adjacent to their recognition sites is well described [82–84]. Epigenetic programming of *NR3C1* gene expression by methylation was demonstrated in numerous studies. Especially, the influence of maternal behaviour, grooming, separation or depression on methylation of the *NR3C1* promoter in the central

nervous system of offspring is of great interest [74,85–89]. Studies dealing with maternal dietary manipulation disclosed a differential methylation of the *NR3C1* promoter in peripheral tissues [10,90]. Most notably, Lillycrop et al. described increased mRNA expression of the glucocorticoid receptor associated with increased promoter methylation in the offspring of mothers fed a protein-restricted diet during gestation [10]. Results of promoter methylation analysis of *PPARα* correspond with the results by Lillycrop et al. [11] who found only slight changes in *PPARα* promoter methylation between 4% and 10% in offspring of protein-restricted rat dams. Our data suggest that methylation of distinct CpG sites within the *NR3C1* and *PPARα* promoter region is involved in alteration of gene expression caused not only by prenatal protein restriction but also by protein excess.

CpG sites in the 5′-flanking regions of *CYP2C34* and *PGC1α* showed high methylation level of up to 80%. In contrast, in CpG island-associated genes, *HMGCR*, *INSR*, *NR3C1* and *PPARα* CpG sites showed no or very low level of methylation. This is consistent with the finding that promoter-associated CpG islands are usually un- or hypomethylated, while single CpG sites scattered throughout the genome are hypermethylated [91–93]. The single CpG sites in the *CYPC34* 5′-flanking sequence showed significantly increased methylation level in LP compared to CO animals at dpn1. The methylation level of *CYP2C34* at dpn1 is consistent with data from neonatal CD-1 mice, reaching a methylation level of up to 80% in the 5′-flanking regions of hepatic *CYP2D9* and *CYP2A4* genes in the liver [94]. Additionally, methylation level was positively correlated with transcript amount. This finding is inconsistent with the idea of gene repression by DNA methylation in the 5′-flanking regions of genes [95]. Nevertheless, several studies revealed the transcriptional control of CYP 450 family genes by methylation of CpG sites within their 5′-flanking regions [94,96,97]. For example, direct *in vitro* methylation of the promoter/enhancer CpG sites of *CYP1B1* caused its transcriptional down-regulation [96]. In contrast, the *CYP1A1* gene revealed a highly stable methylation status, suggesting the indirect regulation of *CYP1A1* via the promoter methylation of *CYP1A1* regulatory factors [97].

HMGCR and *INSR* showed no methylation at CpG sites in the 5′-flanking region, indicating no influence of DNA methylation on the transcriptional regulation of these genes in the porcine protein malnutrition model. In fact, the expression of HMGCR is largely regulated by posttranslational mechanisms. Hence in the liver, HMGCR is one of the downstream targets directly phosphorylated by AMP-activated protein kinase (AMPK) [98]. INSR content can be regulated post-transcriptionally by insulin [55,57]. Furthermore, post-translational modifications of the INSR protein by serine phosphorylation or binding to inhibiting proteins, e.g., PC-1 (ectonucleotide pyrophosphatase/phosphodiesterase 1) as well as SOCS (suppressor of cytokine signaling) and GRB-IR (growth factor receptor-bound protein), can affect the activation of INSR [99,100].

Only a few studies have focused on the influence of high-protein diet on metabolism yet [101]. Thus here in a porcine model of gestational protein supply, we can clearly show that not only protein restriction but also an excess of maternal protein supply affects the gene expression as well as the methylation level of key metabolic genes *HMGCR*, *INSR*, *NR3C1*, *CYP2C34*, *PPARα* and *PGC1α*. In summary, the present study revealed an influence of gestational protein restriction as well as excess on hepatic gene expression of *NR3C1*, *PPARα*, *INSR*, *PGC1α* and *CYP2C34*. Differences in the gene expression of *HMGCR*, *INSR*, *NR3C1*, *CYP2C34*, *PPARα* and *PGC1α* suggest the gene-specific and -sensitive effects of the maternal diet during gestation on the regulation of offspring gene expression. Furthermore, the DNA methylation status of distinct CpG sites in the 5′-flanking region of *NR3C1*, *PPARα* and *CYP2C34* between the offspring of control and the offspring derived from gilts with restricted or gilts with excess protein supply during pregnancy was altered. Moreover, the methylation status of *NR3C1* and *PPARα* in LP offspring was negatively correlated with transcript amounts, implicating an involvement of methylation at the transcriptional control of porcine hepatic *NR3C1* and *PPARα* which seems to allow subtle as well as rapid alterations of gene expression.

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