Somatic cytochrome *c* (*CYCS*) gene expression and promoter-specific DNA methylation in a porcine model of prenatal exposure to maternal dietary protein excess and restriction

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Abstract

There is growing evidence that maternal nutrition during gestation has an important effect on offspring development as well as on their gene expression with long-term effects on the metabolic state. A potential mechanism forming long-lasting gene expression patterns is DNA methylation of cytosine in CpG dinucleotides within the promoter region of distinct genes. There has been special focus on mitochondrial dysfunction by prenatal malnourishment over the recent years. To this end, we investigated the gene expression of somatic cytochrome *c* (*CYCS*), an important member of the respiratory chain, in a porcine model of gestational protein over- and undersupply at 94 d post-conception and 1, 28 and 188 d of age, and analysed the association with the DNA methylation status within the *CYCS* promoter. Gene expression on day 1 *post natum* showed a significant increase in the low protein (LP) group ($P=0.0005$) and a slight increase in the high protein (HP) group ($P=0.079$) compared with the control (CO) group in the liver. The mean of the methylation level over forty-seven CpG sites from nucleotide (nt) -417 to -10 was significantly decreased in the LP ($P=0.007$) and HP ($P=0.009$) groups compared with that in the CO group. Excess and restricted protein supply during pregnancy led to hypomethylation of a number of CpG sites in the *CYCS* promoter, including those representing putative transcription factor-binding sites, associated with elevated expression levels. However, the impact of the low-protein gestation diet is more pronounced, indicating that the offspring could better adapt to excess rather than restricted protein supply.

Key words: Protein restriction: High-protein diet: Gestation: Maternal diet: Fetal programming: Pigs: Intra-uterine growth restriction

In rodent models and in humans, the impact of gestation diets on the offspring's phenotype was shown experimentally and epidemiologically. The underlying programming of fetal development is seen as a risk factor for degenerative diseases in adulthood, including the metabolic syndrome. The fetal programming of adult disease hypothesis has been summarised[1–3]. This relationship was considered as a result of fetal adaptations to intra-uterine malnutrition leading to decreased birth weight and a higher risk for adult disease, which, in the case of a discrepancy between fetal and adult nutrition, leads to increased sensitivity to metabolic disorders[4].

In recent years, there has been increasing interest in intra-uterine programming of traits through maternal nutrient deficiency or excess in livestock[5,6]. Among the livestock species, pigs most prevalently suffer from intra-uterine growth retardation as a result of deficient fetal nutrient supply[5,6]. Piglets with below average birth weight develop reduced muscle growth but increased fat deposition, resulting in impaired meat quality traits with tremendous effects on farm animal production and welfare[7].

There is evidence that intra-uterine malnutrition can have long-lasting effects on the gene expression of the fetus and

therefore can extensively affect the phenotypic outcome of the progeny[8,9]. In rodents, gestational protein restriction is a well-established animal model for the study of nutrient–gene interactions in the offspring[10,11]. Epigenetic effects manifesting during crucial pre- and perinatal developmental stages are increasingly seen as underlying effects of fetal programming[12]. Epigenetic effects involve molecular mechanisms leading to heritable changes in gene expression without nucleotide (nt) sequence alterations[13]. DNA methylation is a well-investigated mechanism of epigenetic gene regulation in which a methyl group is transferred to the 5′ position of genomic cytosine in CpG dinucleotides by DNA methyltransferases[14]. CpG dinucleotides are not equally distributed throughout the vertebrate genomes but accumulate in CpG-rich areas of DNA termed as CpG islands[15,16]. CpG islands are usually hypomethylated and associated with the promoter regions of protein-coding genes[17].

The consequences of maternal protein restriction on the mitochondrial function of the offspring were recently described in rodent models suggesting a role for nutritional mitochondrial programming in the development of mitochondrial dysfunction[18,19]. The process of oxidative phosphorylation taking place in the mitochondria is an important part of energy metabolism and is essential for ATP synthesis. Somatic cytochrome c, encoded by CYCS, is an integral membrane protein occurring in the mitochondrial intermembrane. It acts as an electron transfer protein in the respiratory chain of oxidative phosphorylation transferring one electron from cytochrome bc1 complex to cytochrome c oxidase. Cytochrome c is also involved in apoptosis when released into the cytosol as a coactivator for caspase-3 activation because it binds the apoptotic protease activating factor 1 thus activating downstream effector caspases[20–23].

Based on its function in cell energy metabolism as well as an apoptotic marker, CYCS was chosen as a candidate gene to investigate the influence of gestational protein restriction on mRNA expression and its relationship with the methylation status of the promoter-associated CpG island in a porcine model.

Experimental methods

Animals, diets and sample collection

The 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 Mecklenburg-Vorpommern (Germany). The animal experiment was performed as described previously[24]. In brief, primiparous German Landrace (DL) sows were inseminated at the age of 8 months. The gilts were randomly assigned at mating to the three diet groups and fed these diets throughout gestation: control (CO) group containing 12·1% crude protein (% of DM); high protein (HP) group with 30% crude protein; low protein (LP) group containing 6·5% crude protein, corresponding to a protein:carbohydrate ratio of 1:5, 1:1·3 and 1:10·4, respectively. The diets were formulated to be isoenergetic (13·6 MJ metabolisable energy/kg)[24]. Within 36 h after birth, the piglets were cross-fostered by two to four parity sows that were fed a standard diet during pregnancy. During lactation, a single standard lactation diet was fed (Provital LAC R.325·0; Trede & von Pein, Dammfleth, Germany) with 15·5% crude protein, 0·95% lysine, approximately 13·0 MJ metabolisable energy/kg. After weaning at the age of 28 d, the offspring were fed ad libitum a standard diet for growing and finishing pigs until the end of the experiment (age 185 d). In the present study, the liver samples of offspring derived from a subset of sows of the three diet groups were collected at gestational day 94 ($n_{offspring}$, sixteen per diet group), at birth (dies post natum (dpn)1; $n_{offspring}$, ten per diet group), at weaning (dpn28; $n_{offspring}$, sixteen per diet group) and at dpn185 (finisher pig; $n_{offspring}$, sixteen per diet group) (Fig. 1). At gestational day 94, the fetuses were developed from six sows per diet group by caesarean section. All the liver samples were immediately frozen in liquid N_2 and stored at $-80°C$ until DNA and RNA extraction. Husbandry and slaughtering conditions have already been described in detail[25].

The body weights at gestational day 94 of the fetuses analysed in the present study did not differ among the three diet groups. However, when scoring all the fetuses derived from the complete experiment, HP and LP offspring were growth restricted compared with the CO offspring, but litter size did not differ[24]. At birth, the piglets of the LP and HP groups were significantly lighter than the piglets of the CO group (1·22 (SEM 0·05) kg and LP, 1·06 (SEM 0·05) kg; P=0·033); also piglets of the HP group were lighter than the piglets of the CO group, but that differences were not significant for the animals used in this analysis. Taking into account all the animals of the experiment, the HP and the LP diets led to significant growth retardation at dpn1. This difference did not persist until weaning at dpn28. Also, finisher pigs at the
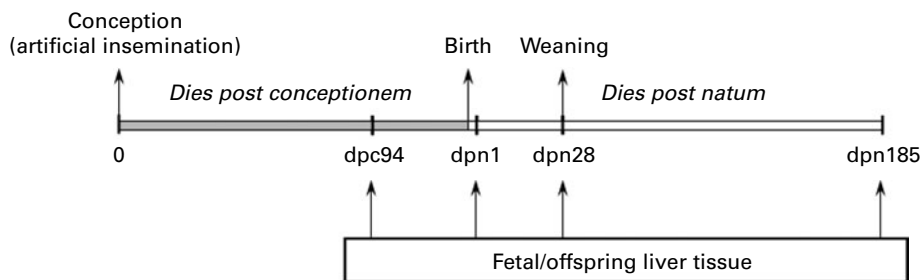

Fig. 1. Time bar of the experimental procedure. Nullipara gilts were artificially inseminated (day 0). Samples of offspring liver tissue were taken at conceptional day 94 as well as at postnatal days 1, 28 and 185. dpc, Dies post conceptionem; dpn, dies post natum.

age of 185 d showed no significant differences in body weight dependent on the diet group[25].

DNA and RNA isolation

The liver samples were powdered by mortar and pestle in liquid N_2. For DNA isolation, phenol–chloroform extraction was performed using Eppendorf Phase Lock Gel Tubes (Eppendorf, Hamburg, Germany). After precipitation, the DNA pellets were dissolved in 500 µl Tris-EDTA (TE) buffer, and concentration and quality were checked by a spectrophotometer (ND1000; Nano Drop Technologies, Wilmington, DE, USA). DNA was stored at -20°C.

RNA was isolated by TRIzol, phenol–chloroform extraction and cleaned with the RNeasy Mini Kit and the RNase-Free DNase Set (Qiagen GmbH, Hilden, Germany) following the supplier's protocol. RNA was quantified spectrophotometrically and stored at -80°C.

RT and quantitative real-time PCR

First-strand complementary DNA was synthesised from 2 µg of total RNA with random primers (Fermentas GmbH, St Leon-Rot, Germany), oligo d(T) 13VN (Sigma-Aldrich, Munich, Germany) and the SuperScript™II Reverse transcriptase Kit (Invitrogen, Darmstadt, Germany) following the manufacturer's guide. Real-time quantitative PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany). The amplification was conducted in duplicate according to the supplier's instructions. The reactions were performed in a final volume of 10 µl using 5·0 µl of LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH), 2·0 µl of Aqua dest, 10 µM of each primer and 40 ng of complementary DNA. The temperature profiles comprised an initial denaturation step at 95°C for 10 min and forty 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. After the completion of the amplification protocol, all samples were subjected to melting curve analysis and gel electrophoresis. For all the assays, the threshold cycles were converted to copy numbers using a standard curve generated by amplifying the serial dilutions of an external PCR standard (10^2–10^7 copies). The primers were obtained from Sigma-Aldrich. The primer sequences for *CYCS* are given in Table 1. Normalisation of the variation in RT-PCR efficiency and initial RNA input was performed using *RPL32* and *RPL10* genes as internal standards by dividing the calculated mRNA copy numbers by a mean normalisation factor derived from the expression of the reference genes (transcript amount).

Porcine CYCS promoter analysis and CpG island identification

The genomic sequence of porcine *CYCS* was obtained from Ensembl Pig FPC map and NCBI nucleotide database and GenBank: FP236676·1_00 902; NC_010460·1 (www.ncbi.nlm.nih.gov; accessed November 2010). For multiple alignment of the *CYCS* 5′ upstream region, the following genomic sequences of human (GenBank: NC_000007·13), bovine (GenBank: NC_007302·4), canine (GenBank: NC_006596·2) and murine (GenBank: NC_000072·5) species were taken from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/; accessed July 2010) and aligned using Mulan[26]. The 5′-flanking region was checked for CpG islands and putative transcription factor-binding sites (TFBS) using EMBOSSs CpGPlot[27] and MultiTF software tools[26], respectively.

Bisulphite sequencing of porcine CYCS promoter

Genomic DNA from newborn piglets (dpn1) was treated with bisulphite by the EZ DNA Methylation Gold Kit (Zymo Research Corporation, Irvine, CA, USA) according to the supplier's instructions. The converted DNA was amplified by semi-nested PCR. The primers that are used are given in Table 1. Nested PCR was performed as follows using Ampli-Taq Gold® DNA Polymerase (Applied Biosystems, Darmstadt, Germany): First PCR: 94°C at 4 min; 94°C at 30 s, 56°C at 40 s, 72°C at 1 min × 40; 72°C at 8 min. Second PCR: 94°C at 4 min; 94°C at 30 s, 58°C at 40 s, 72°C at 1 min × 40; 72°C at 8 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen GmbH). The extent of methylation was determined by direct sequencing of PCR fragments (amplified by using bisulphite-treated DNA as a template) by Qiagen GmbH as follows: all sequencing reaction mixes were based on the BigDy 3·1 Terminator chemistry (Applied Biosystems). Template amounts of 5 ng/100 bases of fragment length and 10 pmol primer/reaction were used.

Table 1. List of primer pairs used for sequencing, quantitative real-time (RT) PCR and bisulphite direct sequencing (BSP)

Primer	5′–3′ sequence	Amplicon (bp)	GenBank resource
CYCS-RT-FP	TCCCCTCCTACAGAGATGGTT		
CYCS −RT-RP	ATGAGATAGCAAAGGGATCGT	165	NM_001129970·1
RPL32-RT-FP	AGCCCAAGATCGTCAAAAAG		
RPL32-RT-RP	TGTTGCTCCCATAACCAATG	165	NM_001001636·1
RPL10-RT-FP	CTGTGTTCGTCTTTTCTTCC		
RPL10-RT-RP	TCATCCACTTTTGCCTTCT	199	NM_001044543·1
CYCS -BSP-FP1	TATAAATGATAGGAGAGGGAAGG		
CYCS -BSP-RP1	CCCTCAAAACTTAACTTCCC	894	FP236676·2
CYCS -BSP-FP2	AGTTTTAGGTGTGTTTAAGTTGG		
CYCS -BSP-RP2	CCCTCAAAACTTAACTTCCC	581	FP236676·2

FP, forward primer; RP, reverse primer.

The reactions were cycled in a GeneAmp PCR System 9700 (Applied Biosystems) and purified using DyeEx™ (Qiagen GmbH). Data collection was carried out on a 3730xl DNA Analyser (Applied Biosystems) equipped with 50 cm capillary arrays and POP 7™ polymer. After data collection, the raw data channels from the result files that were generated (.ab1) were processed using custom-built software developed at Qiagen GmbH. Data processing involved 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 corresponded to variable positions of CpG sites were identified by comparison of the obtained sequence with a reference sequence. The ratios of methylated *v.* unmethylated species (C/T in forward reads and G/A in reverse reads) were reported as percentage methylation. The values of the signal:background ratio (S:B) were used as a quality control of quantification. S:B ratios of 20 and above were considered as high-quality samples and used for the quantification of the methylation status. Furthermore, in order to validate the procedure of measurement of the proportion of methylation at the various CpG sites within each fragment – including the steps of bisulphite treatment, direct sequencing and evaluation of electropherograms – non-methylated and 100 % methylated DNA samples were generated. For the generation of unmethylated samples (0 %), genomic DNA was amplified using REPLI-g UltraFast Mini Kit (Qiagen GmbH) and purified by phenol–chloroform extraction. To produce 100 % methylated samples, genomic DNA was treated with the CpG Methyl transferase Kit (M.SssI 20 000 units/ml; New England Biolabs, Inc., Frankfurt,

Germany) and purified by the phenol–chloroform extraction. Then, the purified 0 and 100 % methylated DNA were treated with bisulphite and used for bisulphite-specific PCR as described earlier. The amplicons of both the treated DNA samples were mixed in different ratios (10–90 %) for direct sequencing by Qiagen Sequencing Services. The algorithm was developed and the procedure was previously validated[28,29].

Statistical analyses

Statistical analyses were performed with SAS 9.2 software package for Windows (SAS Institute, Inc., Cary, NC, USA). Differences in the level of expression of CYCS at each time point, *dies post conceptionem* 94, dpn1, dpn28 and dpn185, measured by real-time RT-PCR and differences in the level of DNA methylation at dpn1 measured by bisulphite sequencing between samples of the offspring of the respective diet groups were evaluated by ANOVA. The 'general linear models' procedure (SAS Institute, Inc.), i.e. the method of least squares to fit the general linear models, and a *post hoc* Tukey–Kramer multiple comparison procedure were used. Dietary group (CO, HP and LP), class of birth weight (light and heavy), sex (female and male) and sow nested within the diet group were considered as fixed effects. Weight at tissue collection was included as a co-variable. Statistical dependence between methylation status and real-time PCR data was estimated using Spearman's rank correlation coefficient. Values are given as least squares means with their standard errors. A *P* value of < 0.05 was considered to be statistically significant.
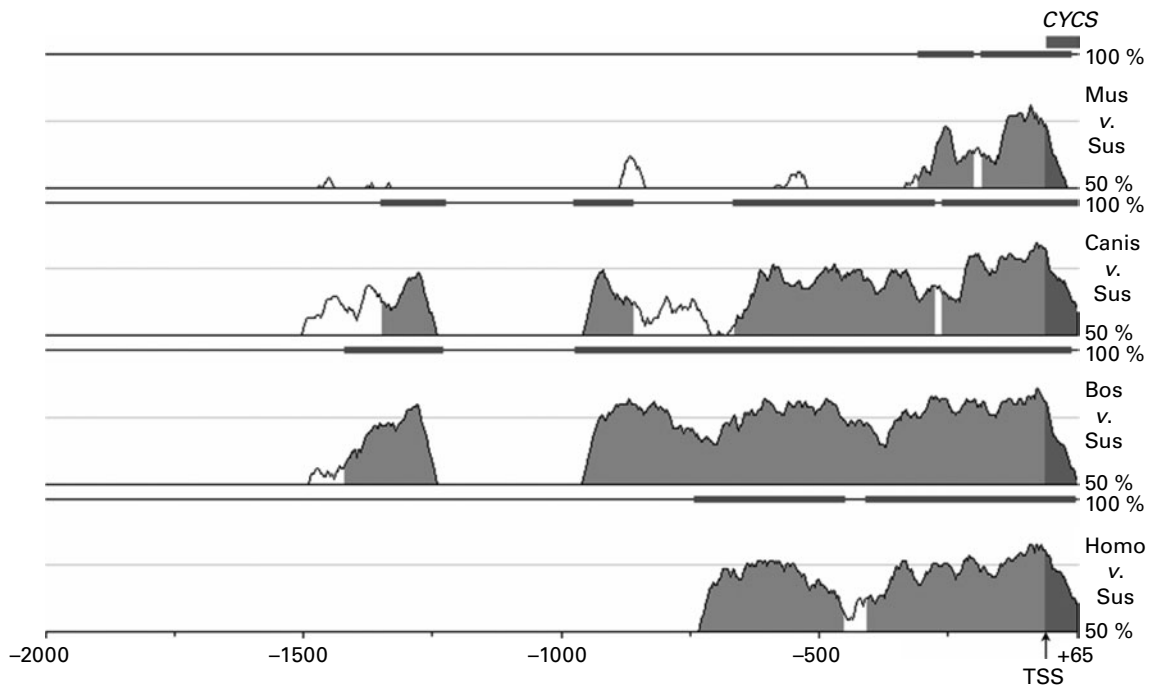

Fig. 2. The Mulan sequence conservation profile for porcine, human, bovine, canine and murine 5′-flanking region of *CYCS*. The horizontal axis displays the input sequences. Evolutionary conserved regions were detected using a threshold of at least 70 % identity over 100 bp and are depicted as dark bars above each pairwise alignment. The 5′-untranslated region (nucleotide (nt) −2000 to nt +65; dark grey) is presented. The *y*-axis shows the percentage similarity of porcine 5′-flanking *CYCS* orthologue to human, bovine, canine and murine sequences. TSS, transcription start site; Mus, murine; Sus, porcine; Canis, canine; Bos, bovine; Homo, human.

Results

Comparative sequence analysis of putative porcine CYCS promoter and CpG island identification

A 2065 bp fragment of 5′-flanking region including exon 1 (nt −2000 to +65 relative to transcription start site) of porcine *CYCS* was aligned to its human, bovine, canine and murine orthologues. The multiple alignment analysis revealed the presence of evolutionary conserved regions with >70 % identity over 100 bp. The Mulan sequence conservation profile is

represented in Fig. 2. The 5′-upstream region in pigs showed high identity compared with the human, bovine, canine and murine sequences (Fig. 2). Pairwise comparison revealed the bovine sequence as the closest potential evolutionary orthologue to the pig 5′-flanking region supported by the highest pairwise identity and length of evolutionary conserved regions. A MultiTF analysis of bovine and porcine orthologue revealed six multi-conserved specific protein 1 and activator protein 2 TFBS (5′-upstream region from nt −500 to −1; Fig. 3). A CpG plot of a DNA region ranging from nt −2000 to +2300
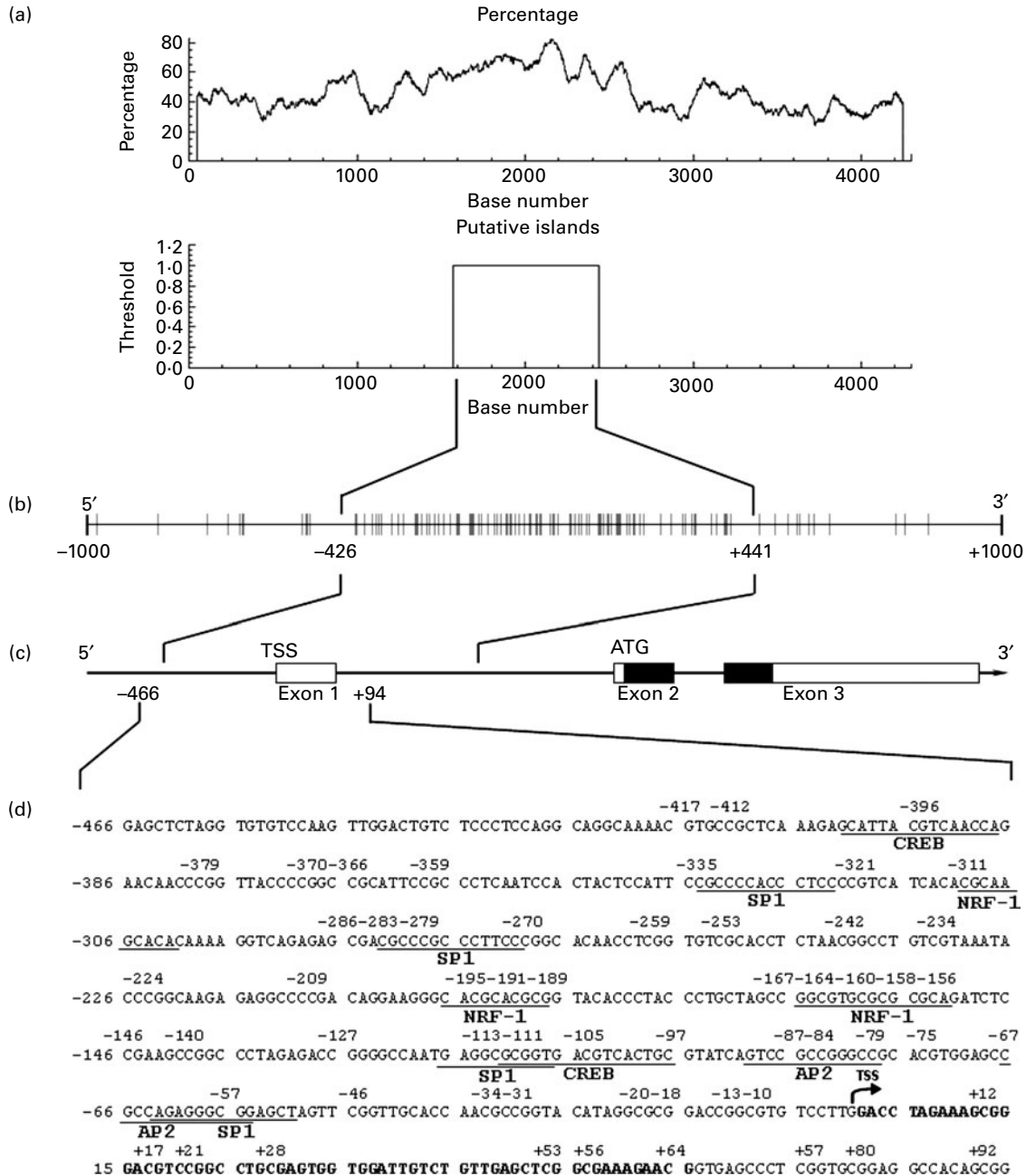

Fig. 3. (a) CpG island identification in the porcine *CYCS* gene (ENSSSCG00000016714) using EMBOSS CpGPlot (settings: observed:expected ratio >0·60, percentage C + percentage G > 60·00, length > 500). (b) CpG density report, CpG sites are indicated by grey bars. (c) Structure of the porcine *CYCS* gene; white boxes, 5′-untranslated region; black boxes, coding sequence; TSS, transcription start site; ATG, start codon. (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; CREB, cAMP response element-binding protein; NRF-1, nuclear respiratory factor 1.
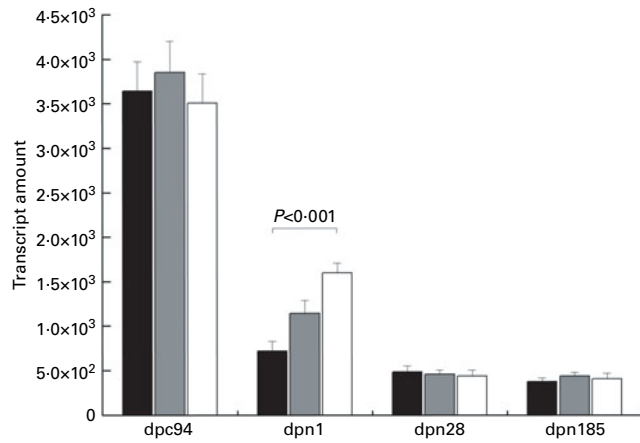
Fig. 4. Transcript amounts of CYCS in the liver at *dies post conceptionem* (dpc)94, *dies post natum* (dpn)1, dpn28 and dpn185. Values are least square means, with their standard errors represented by vertical bars. CO (■), control; HP (▦), high protein; LP (□), low protein.

comprising the whole porcine *CYCS* sequence showed a 867 bp CpG island from nt −426 to +441 that includes exon 1 (Fig. 3). The schematic structure of the *CYCS* gene and position of the CpG island are given in Fig. 3. The CpG island includes ninety-one CpG sites (10·59 % CpG) and shows an overall C + G content of 65·74 % as well as an observed:expected ratio of 0·97.

Gene expression of CYCS

The results of quantitative gene expression analyses are shown in Fig. 4. At gestational day 94, hepatic gene expression of *CYCS* showed no significant differences among the CO, HP and LP groups. In 1-d-old piglets (dpn1), a protein restriction during pregnancy (LP) resulted in a significantly higher gene expression level in the hepatic tissue compared with the CO group ($P=0.0005$). The abundance of *CYCS* transcripts was increased by 55 % in the LP group compared with the CO group. Between the HP and the CO groups at dpn1 gene expression differed at $P=0.079$ (29 % increase in the HP group compared with the CO group). In contrast, the differences were no longer obvious at weaning age (dpn28) and at dpn185 between offspring of the three maternal diet groups. With regard to the ontogenetic development, the amount of *CYCS* transcript decreased significantly between the intra-uterine stage at dpn94 and day 1 after birth. Subsequently, *CYCS* is expressed at a low level at dpn28 and dpn185 (Fig. 4).

Effect of maternal dietary protein level during gestation on methylation of CYCS promoter

Gene expression data of newborn piglets (dpn1) showed significant differences among the CO, HP and LP groups. Thus, we isolated DNA from newborn piglets (dpn1) for methylation analysis. The level of methylation of individual CpG sites of CYCS at dpn1 is shown in Fig. 5. In total, forty-seven CpG sites from the nt position −417 to −10 were investigated. Significant differences between the CO and LP groups were

found for fourteen CpG sites at positions nt −253, −224, −195, −191, −160, −158, −156, −97, −84, −79, −67 and −57 ($P<0.05$). Differences between the CO and HP groups were significant for eight CpG sites at positions nt −253, −224, −195, −158, −140, −84, −79 and −75 ($P<0.05$). Position nt −140 showed a decrease in methylation level in the LP group in comparison with the CO group at $P=0.086$. The mean of the methylation level over the CpG sites from nt −417 to −10 is significantly decreased in the LP ($P=0.007$) and HP ($P=0.009$) groups compared with the CO group. The mean level of methylation of the CpG sites that differed between the LP and CO groups correlated negatively with transcript abundance ($R -0.419$) at $P=0.084$. Also, the mean levels of methylation of the CpG sites differing between the HP and CO groups showed a significant negative relationship between methylation and transcript amount ($R -0.501$, $P=0.041$).

Most of the significant differences in methylation status of single CpG sites were found between nt −253 and the transcription start site (nt +1). Interestingly, the sequence spanning nt −1 to −250 is highly conserved between mice, humans, dogs, cows and pigs (Fig. 3). Moreover, there was no evidence of any polymorphism among the porcine samples analysed in the present study.

Discussion

The influence of maternal nutrition on offspring gene expression and CpG island promoter methylation has been investigated in several genes mainly in rodent models. To the authors' knowledge, the present results show for the first time that hepatic gene expression of *CYCS* in offspring is affected by specific CpG nt methylation in *CYCS* promoter depending on dietary maternal protein level during gestation in the pig. *CYCS* was investigated in the liver tissue because liver is sensitive to maternal nutrient intake during gestation.

CYCS expression of piglets whose mothers were fed a protein-restricted diet (LP) during gestation was significantly increased in comparison with control piglets at dpn1. In the HP group, the expression of CYCS showed a trend towards increased levels. At day 94 of gestation, dpn28 and dpn185, no differences among the three diet groups were obvious, thus implying no long-term effects of maternal protein level on *CYCS* gene expression. However, in neonatals, differences with the gestation diets might indicate a different responsiveness to changes associated with the transition from intra-uterine to extra-uterine life of offspring derived from non-adequately fed gilts compared with offspring of control sows. Gene expression differences in *CYCS* caused by intra-uterine protein undernourishment suggest an interrelation between prenatal nutrition and the outcome of mitochondrial energy metabolism. Furthermore, there is evidence that oxidative stress is associated with intra-uterine growth retardation[30]. Additionally, these results are consistent with data from sheep models of maternal nutrient restriction showing increased cytochrome *c* protein abundance in the liver of 140-d-old fetuses[31] indicating enhanced mitochondrial activity in offspring from nutrient-restricted mothers.
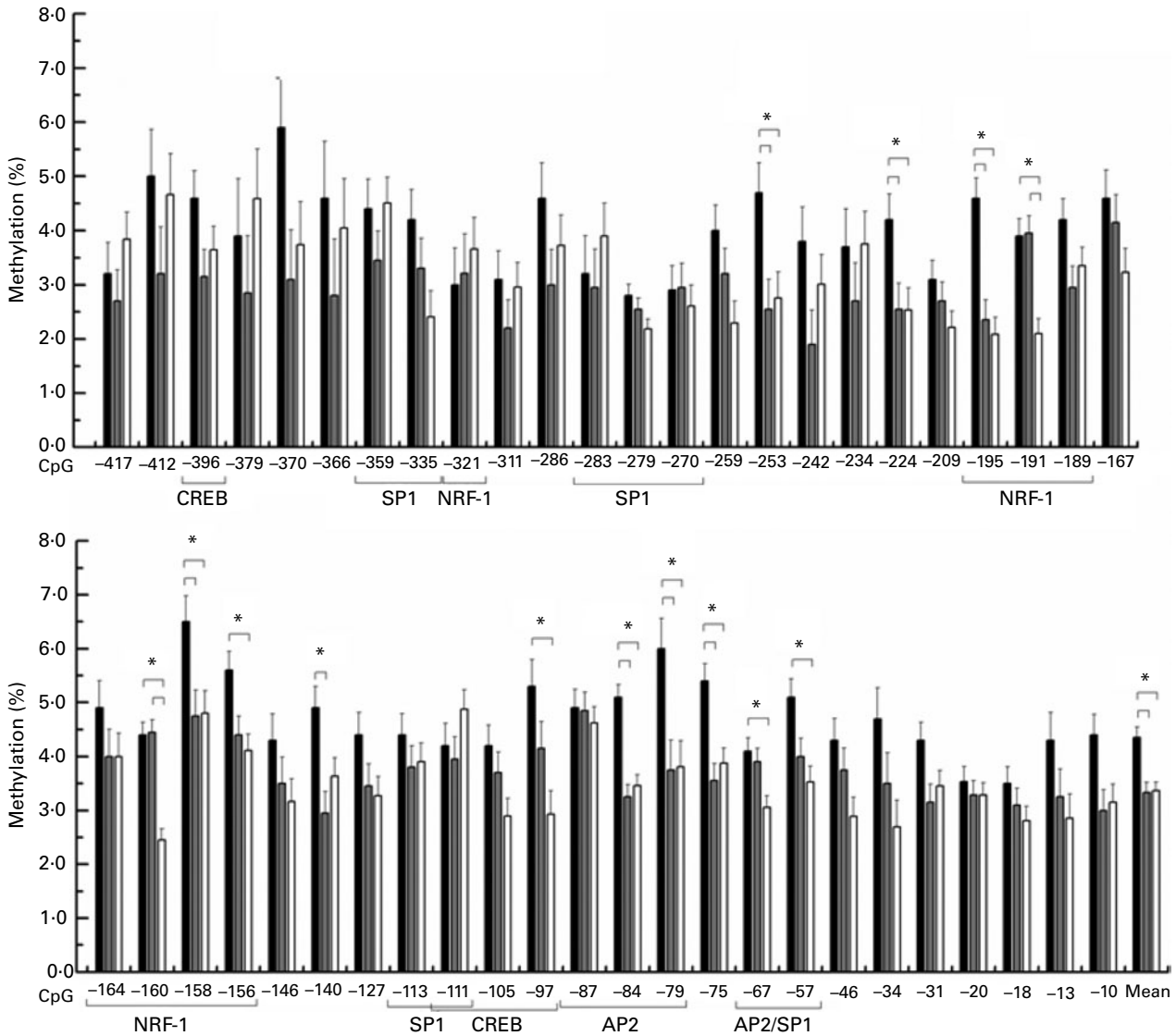
Fig. 5. Methylation of individual CpG sites in the *CYCS* promoter region in the liver tissue of pigs from three diet groups at *dies post natum* (dpn)1. Values are least square means, with their standard errors represented by vertical bars. *Least square means were significantly different ($P < 0.05$). CO (■), control; HP (▨), high protein; LP (□), low protein; NRF-1, nuclear respiratory factor 1; SP1, specificity protein 1; CREB, cAMP response element-binding; AP2, activating protein 2.

Results of gene expression measurement may reflect the different offspring nutritional requirements in the course of pre- and perinatal development. In the last trimester of gestation, fetuses show a tremendous growth and weight gain with increasing protein accretion of up to 4·63 g/d[32,33]. Thus, during the last period of gestation, fetuses are exceedingly susceptible to maternal protein restriction, developing metabolic adaptations to their nutritional situation. Therefore, we assume that differences in body weight and *CYCS* expression were not yet obvious at *dies post conceptionem* 94 since the fetuses were able to cope with maternal protein restriction in the LP group. But at dpn1 differences occur due to high fetal requirements of protein during the last quarter of gestation that could not be supplied in the LP group in contrast to the CO group. Thus, the effects were clearly developed at dpn1. Offspring of the HP group was only slightly affected by the maternal diet during gestation

observable in similar birth weight with CO[24] and showing slight but recognisable differences in *CYCS* gene expression compared with the CO group.

Simultaneous to the increase in *CYCS* gene expression in 1-d-old LP piglets, there is a significant increase in methylation of distinct CpG sites in the promoter region of *CYCS* in the LP offspring as well as in the HP offspring. The observed methylation level was quite low and varied between 2 and 10 %. Nevertheless, there were significant differences in CpG methylation of individual CpG sites of the LP and HP groups in comparison with the CO group. Furthermore, CpG sites showing significantly different methylation levels also exhibited a significant negative correlation with the expression of *CYCS*, thus suggesting an involvement of DNA methylation of the promoter CpG island in transcriptional regulation of *CYCS*.

CpG sites in the 5′-flanking region of porcine *CYCS* showing that significantly different methylation levels coincide with

putative TFBS. The location of differentially methylated CpG dinucleotides within putative TFBS indicates a potential regulation of *CYCS* transcription by DNA methylation. Additionally, most of the significant different methylated CpG sites were found between nt −253 and the transcription start site (nt +1) being located in an evolutionary conserved region between mice, humans, dogs, cows and pigs with a conservation identity of ≥70 % (Fig. 2). Thus, this region is supposed to be a functional promoter region playing a role in cytochrome *c* expression control.

In human and rodent cytochrome *c* promoter recognition sites for nuclear respiratory factor 1 (NRF-1), specific protein 1 and cAMP-response element-binding protein were identified previously[34,35]. The discovered NRF-1 site was highly conserved between rats and humans, and is also highly conserved between murine, human, bovine and porcine sequences (Fig. 2). In porcine 5′-flanking region, this conserved NRF-1 site is located at position nt −166 to −153 (Fig. 3) and contains four CpG sites, of which three show significant differences in methylation level between the CO and LP groups as well as in the HP group (nt −160, −158, −156; Fig. 5). These data suggest an influence of DNA methylation at this position on binding of NRF-1. NRF-1 is a transcription factor that is involved in regulation of several genes with mitochondrial respiratory function[36]. *In silico* search for putative NRF-1 binding sites within the porcine 5′-flanking region exhibited two further sites (Fig. 3). Furthermore, two putative cAMP-response element-binding protein sites conserved among pigs, cows and dogs were found, and one of them contained a CpG with significant differences between methylation level of CO and LP (nt −97; Fig. 5). Additionally, recognition sites of specific protein 1 and activator protein 2 were identified in the porcine sequence. Inhibition of transcription factor binding in specific protein 1 and activator protein 2 binding sites by methylation within and adjacent to the recognition site has been described[37–39].

The low variation in the methylation levels <10 % implicates a sensitive transcriptional control via CpG methylation. These findings correspond with the results reported in the literature[40–42]. Thus Lillycrop *et al.*[40] who found slight changes in *PPARα* promoter methylation between 4 and 10 %, and thus attached 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. Nevertheless, significantly differences between the CO and LP groups were still observable. Our data suggest that methylation of distinct CpG sites within the promoter region of *CYCS* is involved in alteration of its expression caused by prenatal protein restriction.

In summary, we show that gestational protein restriction as well as excess lead to significant differences in DNA methylation level of distinct CpG sites in 5′-flanking region of *CYCS* between the offspring of control and the offspring derived from gilts with restricted or excess protein supply during pregnancy. The decrease in methylation level in offspring of LP and HP groups was associated with an increase in *CYCS* gene expression, implicating an involvement of methylation in the transcriptional control of porcine *CYCS* in the liver,

which seems to allow subtle as well as rapid alterations in gene expression. Interestingly, significant hypomethylation of CpG sites in the experimental groups was particularly observed at conserved regions that represent putative TFBS. In general, effects of the low-protein diets on expression and DNA methylation of *CYCS*, a key component of oxidative mitochondrial energy production, as well as fetal growth were more pronounced than the impact of the high-protein diet, indicating that the offspring could better cope with excess rather than restricted protein supply.

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