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RESEARCH PAPER

Maternal protein and folic acid intake during gestation does not program leptin transcription or serum concentration in rat progeny

Agata Chmurzynska · Monika Stachowiak · Ewa Pruszynska-Oszmalek

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Abstract Maternal nutrition during gestation influences the development of the fetus, thereby determining its phenotype, including nutrient metabolism, appetite, and feeding behavior. The control of appetite is a very complex process and can be modulated by orexigenic and anorexigenic mediators such as leptin, which is involved in the regulation of energy homeostasis by controlling food intake and energy expenditure. Leptin transcription and secretion are regulated by numerous factors, nutrition being one of them. The present study was designed to test whether maternal nutrition can permanently affect leptin gene transcription and leptin serum concentration in rat progeny. Moreover, we analyzed whether leptin expression and secretion in response to high-fat postweaning feeding depends on the maternal diet during gestation. Pregnant rats were fed either a normal protein, normal folic acid diet (the AIN-93 diet); a protein-restricted, normal folic acid diet; a protein-restricted, folic acid-supplemented diet; or a normal protein, folic acid-supplemented diet. After weaning, the progeny was fed either the AIN-93 diet or a high-fat diet. Neither maternal nutrition nor the postweaning diet significantly affected *Lep* transcription. High-fat feeding after weaning was associated with higher serum leptin concentration, but the reaction of an organism to the fat content of the diet was not determined by maternal nutrition during gestation. There was no correlation between *Lep* mRNA level and serum leptin concentration. Global DNA methylation in adipose tissue was about 30% higher in rats fed postnatally the high-fat diet ($P < 0.01$). Our study showed that the protein and folic acid content in the maternal diet had no significant programming effect on *Lep* transcription and serum leptin concentration in the rats.

Keywords Leptin · Fetal programming · Protein deficiency · Folic acid supplementation · High-fat diet · Rat

Introduction

Epidemiological observations and studies using animal models have identified characteristics of fetal programming—a form of developmental plasticity by which organisms adapt to their environment in utero. Maternal nutrition during gestation can exert a long-term effect on the progeny's health by regulating the development, physiology, and function of the body (Chmurzynska 2010). The mechanisms of developmental programming are not thoroughly known, but it has been shown that epigenetic mechanisms may contribute to this phenomenon. The maternal intake of nutrients involved in one-carbon metabolism (e.g., sulfur amino acids, folic acid, choline, vitamin B_6) may dictate the establishment of epigenetic patterns in the progeny and thus permanently alter gene expression (Mathers and McKay 2009; Burdge and Lillycrop 2010; McKay et al. 2010). A mismatch between fetal and postnatal nutritional environment may have maladaptive consequences and is considered to be a

A. Chmurzynska (✉)
Department of Human Nutrition and Hygiene, Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland
e-mail: agata@jay.up.poznan.pl

M. Stachowiak
Department of Genetics and Animal Breeding, Poznań University of Life Sciences, Poznań, Poland

E. Pruszynska-Oszmalek
Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, Poznań, Poland

disease-promoting factor (Bateson et al. 2004; Gluckman and Hanson 2008).

Studies of rodent models indicate that fetal undernutrition can determine excess weight gain and adiposity in adulthood (Langley-Evans et al. 2005). The pathogenesis of obesity triggered by nutritional stimuli acting during prenatal life is not fully understood, but programming of appetite may be one of the contributing factors (Muhlhausler et al. 2008). It was shown that exposure to a low-protein diet during prenatal life can modify feeding behavior in the rat and stimulate a preference for high-fat foods (Bellinger et al. 2004; Bellinger and Langley-Evans 2005; Bellinger et al. 2006). However, providing a higher level of folate in the maternal diet prevents this effect (Engeham et al. 2010). It has also been reported that supplementation of protein-restricted diets with folic acid prevented the programming effects of undernutrition on DNA methylation patterns and altered metabolic phenotype (Lillycrop et al. 2005; Burdge et al. 2008).

The regulation of appetite is a complex process based on the interaction of peripheral signals with the central nervous system, in which the hypothalamus plays a pivotal role (Hainerová and Lebl 2010). Feeding behavior can be modulated by several hormones, including leptin—a polypeptide secreted by adipocytes which controls energy balance and body weight by decreasing food intake (Davis et al. 2010; Morris and Rui 2009). Since leptin concentration during fetal and neonatal life may alter hypothalamic structures, it has been proposed that leptin has an influence on the programming of appetite (Döotsch et al. 2004; Bouret 2009; reviewed by Stocker and Cawthorne 2008).

We hypothesize that maternal diet may alter the growth and development of the body and thus determine the long-term pattern of leptin secretion, which in turn may lead to changes in feeding behavior. The aim of the present study is therefore to assess the effect of protein and folic acid content in the maternal diet during pregnancy on *Lep* gene transcription and leptin serum concentration in the rat. The circulating leptin level reflects adiposity and energy balance but is also influenced by dietary fat content, and so we analyze here whether the reaction of an organism to the nutritional environment can be programmed in utero. We therefore test whether *Lep* expression and secretion in response to a postweaning high-fat diet depends on maternal nutrition during gestation.

Materials and methods

The experiment was approved by the local ethics committee for experiments on animals. Rats were housed individually in metabolic cages at a temperature of 20°C with a 12-h light cycle. Twenty virgin female Wistar rats (5 per group) were naturally mated with 8 male rats. We checked for the presence of a vaginal plug daily, after overnight mating. After confirming successful mating, the female rats were assigned to one of four groups: the NP + NF group was fed a normal protein, normal folic acid diet (20% casein and 2 mg folic acid/kg diet, the modified AIN-93 diet); the RP + NF group was fed a protein-restricted, normal folic acid diet (9% casein and 2 mg folic acid/kg diet); the RP + SF group was fed a protein-restricted, folic acid-supplemented diet (9% casein and 5 mg folic acid/kg diet); and the NP + SF group was fed a normal protein, folic acid-supplemented diet (20% casein and 5 mg folic acid/kg diet). The isocaloric diets were based on AIN93—the standard purified diet for growing, pregnant, and lactating rodents (Reeves et al. 1993). In the protein-restricted diet, casein was replaced by wheat starch, and so all diets were isocaloric. Since casein-based diets are deficient in sulfur amino acids, all the diets were supplemented with L-cystine, and the protein-restricted diets were supplemented by a quantity of L-cystine proportional to the protein content (Table 1). At the age of 4 weeks, the offspring of each mother group were randomly assigned to two groups that were fed either the modified AIN-93 diet (AIN groups) or a high-fat diet (HF groups) until the age of 10 weeks (Fig. 1). In the high-fat diet, wheat starch was partly replaced by sunflower oil and lard (Table 1), and in total, this diet contained 39.5% fat by weight. The cumulative 24-h food intake was monitored for all animals during pregnancy, as well as throughout the postweaning period.

At the end of the experimental period, the animals were euthanized. Blood was collected by cardiac puncture. The abdominal fat was dissected, accurately weighted, and immediately frozen and stored at −75°C for further expression analyses.

The total RNA was extracted from visceral fat tissues using Tripure Isolation Reagent (Roche), according to the manufacturer's protocol. Approximately 1 μg RNA was taken for cDNA synthesis. Samples of RNA were incubated with a set of random hexamers (Roche, 0.25 μg/μl) and oligodT$(_{15})$ (Roche, 0.25 μg/μl) at 70°C/10 min. A mixture of dNTP (5.0 mM, Roche), 1 U reverse transcriptase AMV (EUR$_X$, Poland), and 20 U Protector RNase Inhibitor (Roche) was added. After two hours of incubation at 37°C, the AMV enzyme was inactivated at 94°C/5 min. The cDNA was then diluted 3× and stored at −20°C.

The real-time PCRs were performed on a Light Cycler 2.0 (Roche) based on the SYBR Green detection system (Roche). *18S ribosomal RNA* (*18S rRNA*) and *β-actin* (*Actb*) genes were used to normalize *Lep* expression. The reaction mixture (total 10 μl) consisted of 3 μl cDNA,

Table 1 Composition of the prenatal and postnatal diets (g/kg diet)

Dietary ingredient	Diets				
	NP + NF (modified AIN-93)	RP + NF	RP + SF	NP + SF	HF
Casein	200	90	90	200	200
L-cystine	3	1.35	1.35	3	3
Wheat starch	532	6,423.5	643.5	532	207
Sucrose	100	100	100	100	100
Potato starch	50	50	50	50	50
Vitamins	10	10	10	10	10
Folic acid	–	–	0.005	0.005	–
Mineral mix	35	35	35	35	35
Sunflower oil	70	70	70	70	100
Lard	–	–	–	–	295
Total energy* (kcal/100 g diet)	424	400	400	424	604

NP + NF a normal protein, normal folic acid diet; *RP + NF* protein-restricted, normal folic acid diet; *RP + SF* protein-restricted, folic acid-supplemented diet; *NP + SF* normal protein, folic acid-supplemented diet; *HF* high-fat diet

* The total dietary energy was determined by bomb calorimetry

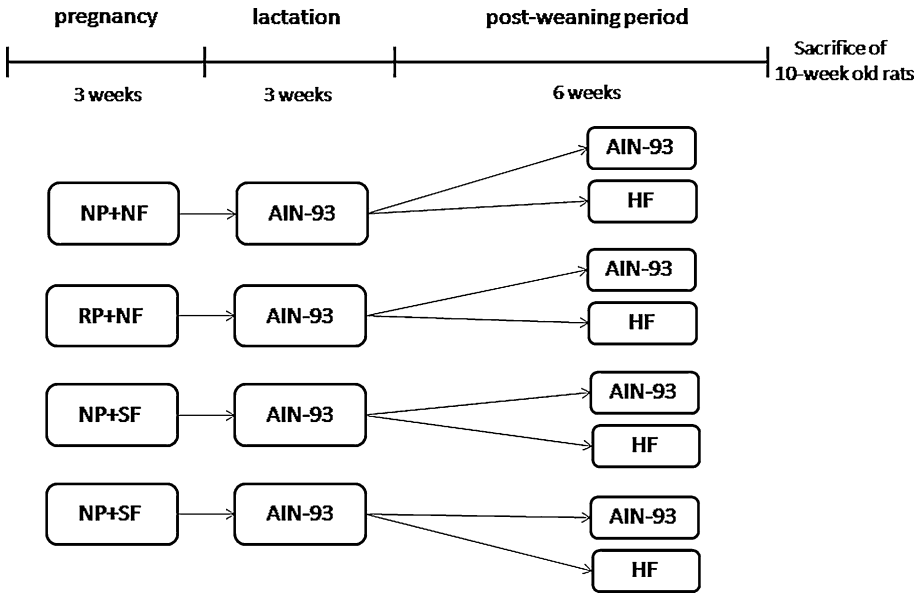

Fig. 1 Schematic flowchart of the experimental design. *NP + NF* a normal protein, normal folic acid diet; *RP + NF* protein-restricted, normal folic acid diet; *RP + SF* protein-restricted, folic acid-supplemented diet; *NP + SF* normal protein, folic acid-supplemented diet; *AIN* the AIN-93 diet; *HF* high-fat diet

0.5 µl LightCycler Fast Start DNA Master SYBR Green I® kit (Roche), 20 µM $MgCl_2$ (*18S rRNA),* 30 µM $MgCl_2$ (*Lep*), or 50 µM $MgCl_2$ (*Actb*), and 0.3 µM forward and reverse primers. The following primers were used: LEP-F: 5′GCC GGT TCC TGT GGC TTT GGT C, LEP-R: 5′GGC GGA TAC CGA CTG CGT GTG; ACTB-F: 5′CTGGGA CGA TAT GGA GAA GAT TTG, ACTB-R: 5′CAT GGC TGGGGT GTT GAA GG; 18S-F: 5′GAC CGG CGC AAG ACG AAC CAG AGC, 18S-R: 5′ AAT AAC GCC GCCGCA TCG CCA GTC. The PCR cycling conditions included predenaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 2 s, and elongation at 72°C for 8 s. Relative quantification of the mRNA level was performed in duplicates based on a Second Derivative Maximum Method (Roche). The abundance of *Lep* gene transcripts was then normalized to a geometric mean of two reference genes (*18S rRNA* and *Actb*) (Vandesompele et al. 2002).

Serum leptin concentration was measured using radioimmunological assay (Rat Leptin Radioimmunoassay Kit; Millipore Corporation, Billerica, MA, USA), according to the manufacturer's protocol. The global DNA methylation shift in the adipose tissue of animals of different maternal dietary groups was determined using Imprint® Methylated DNA Quantification Kit (Sigma–Aldrich), according to the manufacturer's procedure.

The results are presented as group means with their standard errors. Statistical analysis was performed using STATISTICA. The effect of maternal diet and postweaning diets on *Lep* expression was assessed by multi-way ANOVA, followed by a *post hoc* Scheffé's test. $P < 0.05$ was accepted as statistically significant. Correlations were evaluated for statistical significance with Pearson's test.

Results and discussion

Food intake was calculated as group mean calorie daily intake and was normalized for body weight (kcal/day per 100 g body weight), and no differences between maternal dietary groups were found (NP + NF 5.8 ± 0.32; RP + NF 6.06 ± 0.09; RP + SF 6.59 ± 0.27; NP + SF 6.05 ± 0.35 g/day per 100 g body weight). Neither maternal diet during gestation nor postnatal feeding affected body weight in the juvenile rats. Abdominal fat mass was influenced by fat content in the postweaning diet ($P < 0.001$) and was much higher in the high-fat diet–fed groups (17.2 ± 0.9 g) than in the AIN diet–fed groups (12.3 ± 0.8 g).

The relative *Lep* gene expression in each maternal dietary group is presented in Fig. 2a. In all groups, *Lep* gene expression was reduced compared to the NP + NF group, with the lowest level in the progeny of dams fed the protein-restricted and folic acid-supplemented diet (RP + SF). However, none of the experimental factors statistically significantly affected the *Lep* mRNA level. Leptin serum concentration was similar in each maternal dietary group (Fig. 2b), but significantly higher in the rats postnatally fed the high-fat diet ($P < 0.01$). The effect of fat content in the postweaning diet accounted for almost 17% of the overall variance. Similar results were presented by Handjieva-Darlenska and Boyadjieva (2009). They showed that high-fat diet gives rise to hyperleptinemia, which is positively correlated with epididymal fat mass. On the other hand, Ainslie et al. (2000) have shown that a moderately high-fat diet is associated with reduced leptin secretion and increased weight gain.

In the present study, there was no effect of maternal nutrition during gestation on leptin serum level in the progeny. Moreover, no interaction effect between prenatal and postnatal nutrition on leptin concentrations was found. This is in agreement with the results obtained by Engeham et al. (2010). Although they used slightly different experimental diets, they also observed no alterations in leptin serum concentrations upon prenatal protein restriction or folic acid supplementation. These data might suggest that dietary factors involved in one-carbon metabolism in the maternal diet have no programming effect on leptin expression and secretion. However, other elements of the leptin signaling cascade may be involved in adaptation to the intrauterine environment. Manuel-Apolinar et al. (2010) have shown, for example, that food restriction during gestation is associated with an increase in the hypothalamic expression of the leptin receptor gene. On the other hand, a maternal low-protein diet may change the expression of other central or peripheral mediators of appetite, as was shown by Orozco-Sólis et al. (2009). The prenatally protein-restricted animals exhibited hyperphagia, which was accompanied by enhanced expression of orexigenic peptides, agouti-related protein, and neuropeptide Y, and by decreased hypothalamic levels of the anorexigenic peptide proopiomelanocortin.

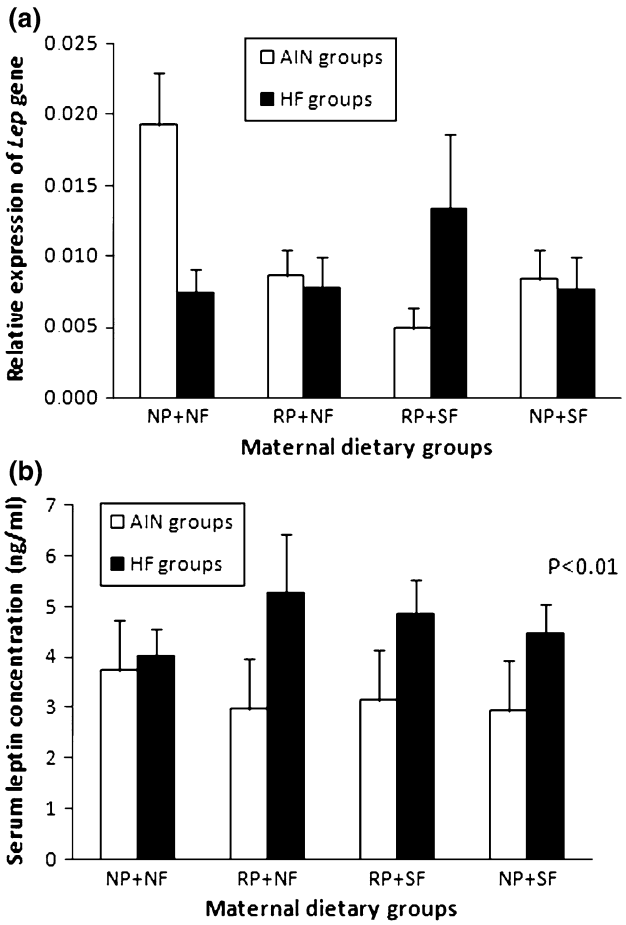

Fig. 2 Effects of prenatal and postnatal diet on: **a** leptin mRNA expression and **b** serum leptin concentration. For the whole pregnancy, dams were fed the following diets: NP + NF; RP + NF; NP + SF; RP + SF. Offspring were fed after weaning the following diets: AIN; HF. The results are presented as mean values with the standard error. $P < 0.05$ is considered statistically significant. *NP* normal protein, *RP* restricted protein, *NF* normal folic acid, *SF* supplemented folic acid, *AIN* AIN-93 diet, *HF* high fat. *The effect of fat content in the postweaning diet

In the present study, the correlations between *Lep* expression, leptin serum concentration, calorie intake, body weight, and abdominal fat weight were also assessed. In some maternal dietary groups, body weight, abdominal fat weight, and calorie intake were positively correlated with serum leptin concentrations ($P < 0.05$)—see Table 2. In all groups except the RP + SF AIN group, calorie intake was also found to correlate with body weight and abdominal fat levels, and the correlation coefficients were over 0.95 and about 0.80, respectively; $P < 0.05$. Leptin gene expression was not significantly correlated with calorie intake. Interestingly, there was no correlation between *Lep* mRNA level and serum leptin concentration. It is well known that

Table 2 Correlation coefficients between serum leptin concentration and body weight, abdominal fat weight and calorie intake in each maternal dietary group

Group	Leptin concentration and body weight	Leptin concentration and abdominal fat	Leptin concentration and calorie intake
NP + NF AIN	0.74	0.76	0.79
NP + NF HF	0.73	0.93	0.76
RP + NF AIN	NS	NS	NS
RP + NF HF	NS	0.89	NS
RP + SF AIN	NS	0.95	NS
RP + SF HF	NS	0.84	NS
NP + SF AIN	NS	NS	NS
NP + SF HF	NS	0.91	NS

$P < 0.05$ was accepted as statistically significant

NS not significant

serum leptin levels decrease on starvation and rapidly increase after feeding (Becker et al. 1995; Thompson 1996). However, variations in leptin transcription level do not entirely account for the changes in the serum leptin concentration, and leptin production is also regulated at posttranscriptional steps (Lee et al. 2007). Moreover, leptin can be stored, secreted, or degraded, and its regulated release from the intracellular pool contributes to the serum leptin level (Lee and Fried 2006). These facts may partly explain the lack of correlation between *Lep* transcription and the leptin serum concentration.

The global methylation status was determined in adipose tissue, and there were no differences found between the maternal dietary groups. This is in agreement with the observations of Engeham et al. (2010). However, methylation shift was observed depending on the type of postnatal diet, with about 30% higher global methylation level being observed in the HF groups, compared to the AIN groups ($P < 0.01$). It has been shown that alterations in the methylation status of gene promoters are associated with the dietary fat content. Methylation of the CpGs near the transcription start site of the melanocortin-4 receptor gene decreases after high-fat feeding (Widiker et al. 2010). On the other hand, leptin promoter was methylated more in cafeteria-fed animals (Milagro et al. 2009). This may suggest that diet-induced changes in methylation are locus-specific.

Summarizing, our study has shown that protein and folic acid content in the maternal diet during gestation does not determine *Lep* mRNA level in adipose tissue and serum leptin concentration in the rat progeny. Moreover, the response to the high-fat diet after weaning is independent of maternal dietary history.

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References

Ainslie DA, Proietto J, Fam BC, Thorburn AW (2000) Short-term, high-fat diets lower circulating leptin concentrations in rats. Am J Clin Nutr 71(2):438–442

Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG, Sultan SE (2004) Developmental plasticity and human health. Nature 430(6998): 419–421

Becker DJ, Ongemba LN, Brichard V, Henquin JC, Brichard SM (1995) Diet- and diabetes-induced changes of ob gene expression in rat adipose tissue. FEBS Lett 371(3):324–328

Bellinger L, Langley-Evans SC (2005) Fetal programming of appetite by exposure to a maternal low-protein diet in the rat. Clin Sci Lond 109(4):413–420

Bellinger L, Lilley C, Langley-Evans SC (2004) Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. Br J Nutr 92(3):513–520

Bellinger L, Sculley DV, Langley-Evans SC (2006) Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. Int J Obes Lond 30(5):729–738

Bouret SG (2009) Early life origins of obesity: role of hypothalamic programming. J Pediatr Gastroenterol Nutr 48(Suppl 1):S31–S38

Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr 21(30):315–339

Burdge GC, Lillycrop KA, Jackson AA, Gluckman PD, Hanson MA (2008) The nature of the growth pattern and of the metabolic response to fasting in the rat are dependent upon the dietary protein and folic acid intakes of their pregnant dams and post-weaning fat consumption. Br J Nutr 99(3):540–549

Chmurzynska A (2010) Fetal programming: link between early nutrition, DNA methylation, and complex diseases. Nutr Rev 68(2):87–98

Davis JF, Choi DL, Benoit SC (2010) Insulin, leptin and reward. Trends Endocrinol Metab 21(2):68–74

Döotsch J, Rascher W, Meissner U (2004) Perinatal programming of appetite control by leptin? Eur J Endocrinol 151(2):159–160

Engeham SF, Haase A, Langley-Evans SC (2010) Supplementation of a maternal low-protein diet in rat pregnancy with folic acid ameliorates programming effects upon feeding behaviour in the absence of disturbances to the methionine-homocysteine cycle. Br J Nutr 103(7):996–1007

Gluckman PD, Hanson MA (2008) Developmental and epigenetic pathways to obesity: an evolutionary-developmental perspective. Int J Obes Lond 32(Suppl 7):S62–S71

Hainerová IA, Lebl J (2010) Mechanisms of appetite regulation. J Pediatr Gastroenterol Nutr 51(Suppl 3):S123–S124

Handjieva-Darlenska T, Boyadjieva N (2009) The effect of high-fat diet on plasma ghrelin and leptin levels in rats. J Physiol Biochem 65(2):157–164

Langley-Evans SC, Bellinger L, McMullen S (2005) Animal models of programming: early life influences on appetite and feeding behaviour. Matern Child Nutr 1(3):142–148

Lee MJ, Fried SK (2006) Multilevel regulation of leptin storage, turnover, and secretion by feeding and insulin in rat adipose tissue. J Lipid Res 47(9):1984–1993

Lee MJ, Yang RZ, Gong DW, Fried SK (2007) Feeding and insulin increase leptin translation. Importance of the leptin mRNA untranslated regions. J Biol Chem 282(1):72–80

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr 135(6):1382–1386

Manuel-Apolinar L, Zarate A, Rocha L, Hernández M (2010) Fetal malnutrition affects hypothalamic leptin receptor expression after birth in male mice. Arch Med Res 41(4):240–245

Mathers JC, McKay JA (2009) Epigenetics—potential contribution to fetal programming. Adv Exp Med Biol 646:119–123

McKay JA, Waltham KJ, Williams EA, Mathers JC (2010) Folate depletion during pregnancy and lactation reduces genomic DNA methylation in murine adult offspring. Genes Nutr 6(2):189–196

Milagro FI, Campión J, García-Díaz DF, Goyenechea E, Paternain L, Martínez JA (2009) High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. J Physiol Biochem 65(1):1–9

Morris DL, Rui L (2009) Recent advances in understanding leptin signaling and leptin resistance. Am J Physiol Endocrinol Metab 297(6):E1247–E1259

Muhlhausler BS, Adam CL, McMillen IC (2008) Maternal nutrition and the programming of obesity: The brain. Organogenesis 4(3):144–152

Orozco-Sólis R, Lopes de Souza S, Barbosa Matos RJ, Grit I, Le Bloch J, Nguyen P, Manhães de Castro R, Bolaños-Jiménez F (2009) Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding. Physiol Behav 96(3):481–492

Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123(11):1939–1951

Stocker CJ, Cawthorne MA (2008) The influence of leptin on early life programming of obesity. Trends Biotechnol 26(10):545–551

Thompson MP (1996) Meal-feeding specifically induces obese mRNA expression. Biochem Biophys Res Commun 224(2):332–337

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Spelman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:1–12

Widiker S, Karst S, Wagener A, Brockmann GA (2010) High-fat diet leads to a decreased methylation of the Mc4r gene in the obese BFMI and the lean B6 mouse lines. J Appl Genet 51(2):193–197