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Effect of maternal dietary supplementation of one-carbon-cycle substrates and co-factors during gestation on hepatic gene expression in pigs at different developmental stages

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Dedicated to

my parents and my advisor

Effekte der Zugabe von Substraten und Kofaktoren des Methionin-Stoffwechsels zur maternalen Diät während der Trächtugkeit auf die hepatische Genexpression von Schweinen in unterschiedlichen Entwicklungsstadien

Deutsche Landrasse (DL)- und Pietrain (PI)-Sauen wurden während ihrer Trächtigkeit mit einer mit Substraten und Kofaktoren des Methionin-Stoffwechsels supplementierten Diät ('MET') oder einer Kontrolldiät ('CON') gefüttert. Anschließend wurde fetales Lebergewebe der Tage 35, 63 und 91 post conceptionem (dpc) und Lebergewebe eines postnatalen Stadiums (158 dies post natum, dpn) auf diät-abhängige differentielle Genexpression überprüft. Die hepatischen Expressionsprofile zeigten jeweils 2055, 641, 2475 und 327 differentiell expremierte Transkripte in DL und 618, 1539, 1592 und 112 differentiell expremierte Transkripte in PI an den Tagen 35, 63 und 91 post conceptionem und dem postnatalen Stadium.

Die konsistent betroffenen Signalwege umfassen die Signaltransduktion über Wachstumsfaktoren, Interzelluläre- und Second-Messenger und Zellkernrezeptoren sowie das Zellwachstum, die Proliferationsentwicklung, den Aminosäure-Metabolismus und, Apoptose. Differentielle Expression zwischen 'CON' und 'MET' wurde mittels real-time PCR für 11 Gene in DL (BHMT, DNMT1, AMD1, IGFBP3, IGFBP5, SHC1, AAK1, SMAD5, MAP3K7, IGF1, SOCS3) und 12 Gene in PI (PFN, Rho, PAK, IGFBP3, IGFBP5, IGF2R, BHMT, DNMT1, MTAP, FOXO1, BCL2, MAFG) bestätigt, die alle aus relevanten kanonischen Signalwegen ausgesucht wurden. Wichtige diätabhängig modulierte Signalwege in beiden Rassen waren u.a. der Methionin-Metabolismus-Signalweg, der IGF-1-Signalweg und der Wachstumshormonsignalweg.

Wir haben sowohl die S-Adenosylmethionin(SAM)- und die S-Adenosylhomocystein (SAH)-Konzentrationen als auch das SAM/SAH-Verhältnis in der Leber von Sauen und Feten von DL und PI bestimmt. Das SAM/SAH-Verhältnis in Feten war in der 'MET'-Gruppe niedriger als in der 'CON'-Gruppe, was in direktem Zusammenhang zur Inhibition von Transmethylierungsreaktionen und im Einklang mit den Ergebnissen zur globalen DNA-Methylierung steht.

Fazit: Supplementation der Gestationsdiät mit Substraten und Kofaktoren des Methionin-Stoffwechsel beeinflusst abhängig vom Entwicklungsstadium und der Rasse sowohl die hepatische Genexpression als auch die mit Wachstum und Fleischqualität zusammenhängenden Merkmale in pränatalen und postnatalen Stadien.

Effects of maternal dietary supplementation of one-carbon-cycle substrates and co-factors during gestation on hepatic gene expression in pigs at different developmental stages

German Landrace (DL) and Pietrain (PI) sows were fed either a gestation diet supplemented with substractes and co-factors of the one-carbon-cycle ('MET') or a control ('CON') gestation diet. Fetal liver tissue of day 35, 63 and 91 post conception (dpc) and a postnatal stage (158 dies post natum, dpn) was subsequently monitored for diet-dependent differential gene expression. The hepatic expression profiles revealed transcripts differentially expressed in DL (2055, 641, 2475 and 327 transcripts) and PI (618, 1539, 1592 and 112 transcripts) on day 35, 63 and 91 post conception and at postnatal stage, respectively. The pathway categories that were consistently affected comprise growth factor signaling, cellular growth, proliferation development, intercellular and second messenger signaling, amino acid metabolism, apoptosis and nuclear receptor signaling. Differential expression between 'CON' and 'MET' was confirmed via quatitative real-time PCR for 11 genes from DL (BHMT, DNMT1, AMD1, IGFBP3, IGFBP5, SHC1, AAK1, SMAD5, MAP3K7, IGF1, SOCS3) and 12 genes from PI (PFN, Rho, PAK, IGFBP3, IGFBP5, IGF2R, BHMT, DNMT1, MTAP, FOXO1, BCL2, MAFG) all of which were selected from relevant canonical pathways. The important canonical pathways modulated by the gestation diets in both breeds included the methionine metabolism pathway, the IGF-1 signaling pathway and the growth hormone signaling pathway. We assessed the hepatic S-adenosylmethionine (SAM) and the S-adenosylhomocysteine (SAH) concentrations as well as the SAM/SAH ratio in sows and fetuses from DL and PI. The SAM/SAH ratio of fetuses was lower in 'MET' than in 'CON', which is closely linked to the inhibition of transmethylation reactions and conforms to results from global DNA methylation. In conclusion, supplementation of gestation diets with substrates and co-factors of the one-carbon-cycle affects hepatic gene expression as well as traits related to growth and meat performance at prenatal and postnatal stages, dependent on the developmental stage and the breed.

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List of abbreviations

5CH3THF	: 5-methytetrahydrofolate
ADP	: adenosine diphosphate
ATP	: adenosine triphosphate
belly score	: belly score 1 lean to 9 fat
bf_13th/14th	: backfat measured at at 13th/14th rib
bf_lumbal	: backfat measured at last lumbar
bf_neck	: backfat measured at neck
BHMT	: betaine-homocysteine methyltransferase
BSAS	: the British Society of Animal Science
bw_abs	: body weight at weaning (28 days of life)
bw_d14	: body weight at 14 days of life
bw_d21	: body weight at 21 days of life
bw_d7	: body weight at 7 days of life
bw_sla	: body weight at slaughter (average 158 days of life)
bw_w12	: body weight at 12 weeks of life
bw_w18	: body weight at 18 weeks of life
cl	: carcass length
cond1mld	: conductivity in Mld at 13th/14th rib 1 h post mortem
cond24mld	: conductivity in Mld at 13th/14th rib 24 h post mortem
cw_cold_le	: carcass weight cold, left half
cw_hot	: carcass weight warm
cw_hot_le	: carcass weight warm, left half
cw_hot_ri	: carcass weight warm, right half
DL	: german landrace
DMG	: dimethylglycine
DNA	: deoxyribonucleic acid
dNTP	: deoxyribonucleotide triphosphate
dpc	: days post conception
DRIP	: drip loss percentage of weight loss after 48 h of Mld samples collected at 24 h post mortem

dTMP	: deoxythymidine monophosphate
dUMP	: deoxyuridine monophosphate
dUTP	: desoxyuridintriphosphate
FAO	: food and agriculture organization
FC	: fold change
FEDNA	: the federal nurses association
GCRMA	: gc robust multi-array average
Gly	: glycine
ham_lenght	: ham length
ham_size	: ham circumference
ITP	: the interventions testing program
LSM	: least square means
mar	: marbling score 1 lean to 5 fat
MAS5	: affymetrix microarray suite version 5
MAT	: methionine adenosyltransferase
meat%	: meat percentage
MH	: malignant hyperthermia
Minolta a	: meat colour 24 h post mortem in Mld at 13th/14th rib; Minolta, redness
Minolta b	: meat colour 24 h post mortem in Mld at 13th/14th rib; Minolta, yellowness
Minolta L	: meat colour 24 h post mortem in Mld at 13th/14th rib; Minolta lightness
Mld	: musculus longissimus dorsi
mRNA	: messenger RNA
MS	: methionine Synthase
MT	: methyltransferase
MTA	: methylthioadenosine
MTAP	: methylthioadenosine Phosphorylase
MTHFR	: methylenetetrahydrofolate reductase
MTOB	: 4-methylthio-2-oxobutanoic acid

MTR1P	: methythioribose-1-PO4
NRC	: the National Research Council
OPTO	: meat colour 24 h post mortem in M. longissimus dorsi (Mld) at 13th/14th rib; OPTO star
PC	: phosphatidylcholine
PC	: phosphatidylcholine
PCR	: polymerase chain reaction
PE	: phosphathidylethanolamine
pH1mld	: pH-value in M. longissimus dorsi (mld) at 13th/14th rib 1 h post mortem
pH1msm	: pH-value in M. semimembranosus (msm) at 1 h post mortem
pH24mld	: pH-value in M. longissimus dorsi (mld) at 13th/14th rib 24 h post mortem
pH24msm	: pH-value in M. semimembranosus (msm) at 24 h post mortem
PI	: pietrian
RMA	: robust multi-array average
RT	: reverse transcription
SAH	: s-adenosylhomocysteine
SAM	: s-adenosylmethionine
SE	: standard errors
Ser	: serine
THF	: tetrahydrofolate

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1. Introduction

Pregnancy is a period of rapid growth and cell differentiation, both for the sow and fetus. It is a period when both are vulnerable to changes in dietary supply, especially of those nutrients that are marginal under normal circumstances. Experimental and epidemiological studies provide many practical means to determine the effects on the developmental fetus physiology that may lead to permanent consequences in its postnatal life. The theory of the "Foetal origins of adult disease" has been described from extensive epidemiological studies, such as marked femine or maternal obesity and this effect has also been studied and reported in multiple species such as pigs (Rehfeldt et al., 1993; Gatford et al., 2003) or sheep (Greenwood et al., 1998). Many studies have been conducted during the last decade on amino acid requirements of sows (NRC, 1998). Methionine, folate, vitamin B12, vitamin B6, choline and betaine are components and co-factors of the one-carbon cycle that function in multiple physiological pathways. In particularly, prenatal maternal environmental exposure to diets high in folate, vitamin B12, choline and methionine are inter-related through their roles in methyl metabolism and have been impacted to homocysteine in mammals (Reik et al., 2001; Ulrey et al., 2005; Friesen et al., 2008) which drives a transient increase in S-adenosylmethionine and S-adenosylhomocysteine by donated methyl groups. The methyl groups are needed for all biological methylation reactions and are substrates for SAM methyl transfer reactions of DNA, RNA, proteins, neurotransmitters and phospholipids. Methylation of DNA is crucially involved in controlling gene expression, cell differentiation, x-chromosome inactivation and imprinting (Bird, 2002; Troen et al., 2003)

Liver plays a central role in the regulation of the metabolic status, the partitioning of nutrients and the expenditure of energy. Ultrastructural studies of pig fetal livers show that its development can be divided into three periods: a period of differentiation [18–40 days postconception (dpc)], a period of metabolic activity (40–80 dpc), and a period of glycogen accumulation (80–113 dpc) (Bielańska-Osuchowska, 1996). Prenatal hepatic expression profiles revealed functional networks that reflect the stage-specific major hepatic activities and indicated, that Pietrain tend to exhibit higher expression of genes involved in changes and development of tissues and cells whereas German Landrace tend to show higher

transcript abundance of genes involved in intracellular energy and metabolite conversion. These trends are even visible at prenatal stages of development that are targets of nutritional programming (Ponsuksili et al., 2007).

Affymetrix microarray has become a powerful tool for detecting differential expression in liver tissue due to body composition, developmental stage, and breed (Ponsuksili et al., 2007), in the analysis of immune response (Afonso et al., 2004), host pathogen interaction (Moser et al., 2004) and in muscle tissue that affect water-holding capacity of pork (Ponsuksili et al., 2008).

We aim at demonstrating differential expression of genes sensitive to fetal programming promoted by maternal diets in a porcine model. Pigs of two breeds, Pietrain and German Landrace - known to differ in lean to fat ratio and exhibiting differences of hepatic expression profiles - were used in an experiment where a gestation diet supplemented with substrates and cofactors of the one-carbon cycle and a control diet were fed to sows throughout pregnancy in order to evaluate genetic-based variation of susceptibility to this mechanism by using Affymetrix microarray and subsequent statistical and bioinformatics evaluation of the expression profiles. Knowledge of how nutrients target specific regulatory genes and thereby influence the overall energy homeostasis will enable to elucidate molecular routes of genotype-environmental (nutritional)-interaction and to provide more precise diet recommendations and appropriate treatments of metabolic diseases.

2. Literature review

Pregnancy is a period of rapid growth and cell differentiation, both for the mother and the fetus. Consequently, it is a period when both are vulnerable to changes in dietary supply, especially of those nutrients that are marginal under normal circumstances. It is known that maternal nutrition, and thus intake of nutrients for the oocyte and in the developing embryo or fetus, is one of the most important environmental factors for the development of offspring (Maloney and Rees, 2005) and leads to permanent consequences in its postnatal life. This effect was shown experimentally and in epidemiological studies in humans (Osmond et al, 1993; Hornstra et al. 2005; Kunz and King, 2007) and model animals such as pigs (Rehfeldt et al., 1993; Gatford et al., 2003) or sheep (Greenwood et al., 1998). For instance, existing literature in pigs indicates that the variation in growth performance after birth may be largely determined, and essentially pre-programmed, during foetal development in the uterus (Dwyer et al., 1994; Gatford et al., 2003).

The following paragraphs of the Literature review focus on some basic aspects and key issues of nutritional/fetal programming:

- Effects of the gestation diets on the offspring may differ at various developmental stages of the fetuses and age of the sows due to changing nutritional requirements. There are increasing evidences that the feed intake after mating plays a major role on embryo survival (Jindal et al. 1996; Novak et al. 2003). The peak of embryo death occurs during the first month of pregnancy, and controlled feed intake is important to reduce mortality of embryos (Geisert and Schmitt 2002; Novak et al. 2003). Manipulation of feed intake before mating may affect embryo survival through changes in follicular development by altering oocyte quality (Ferguson et al. 2003). Ulrey et al. (1965) showed that the rate of growth of porcine fetal tissues at various stages of gestation. In particular, porcine fetal growth accelerates during the second half of pregnancy (Knight et al., 1977; Wu et al., 1999; Pond and Mersmann, 2001). Nutritional requirements of the sows also change along life time. Maternal anabolism is greater in young than in older sows, because they have a higher growth path of the mother as adults (Young et al., 2005)

- Impact of maternal nutrition on gene expression has become an area of important research. Various nutrients and experimental diets were studied. In particular amino acid supply is considered because the amino acids have many important functions; their homeostasis has to be finely maintained. However, the amino acid can be affected by certain nutritional conditions and various forms of stress. Therefore, mammals have to adjust the physiological functions involved in adaptation to the availability of amino acids. Methionine is critical in the context of programming as a donor of methyl groups for DNA methylation which is the main epigenetic mechanisms of fetal programming. The methionin metabolism involves a number of other substrats and co-factors that were considered in this experiment.

- Recently progress has been made in the understanding the mechanisms involved in the control of gene expression in mammals in response to modulation of nutritional supply. DNA methylation was recognized as a major mechanism, of epigenetic regulation of gene expression and represents the molecular equivalent of the metabolic memory that is established in the framework of nutritional/fetal programming.

- Breed or genetic make-up may affect the response to various gestation diets at the level of the organismal phenotype but also at the level of the transcriptome. Prenatal expression profiling revealed hepatic functional networks that reflect the main activities of the liver. Hepatic expression profiling at several ontogenetic stages indicated, that Pietrain tend to have higher expression of genes involved in the changes and development of tissues and cells, while the German Landrace tend to show a greater abundance of the transcription of genes involved in transduction and transformation of energy metabolite. These trends are still visible in various stages of prenatal development, which are the targets of nutritional programming (Ponsuksili et al., 2007).

2.1 Maternal and fetal growth and nutritional requirements

There are many factors including genetics, nutrition, and gestational stage that are the major determinants of growth rates in fetal pigs (Anthony et al., 1995; Fall et al., 2003). The energy requirement for growth of the mother can be calculated by the expected amount used for maintenance and growth depending on its composition, which in turn varies with genetic lines, the requirements for the recovery of the mother sow and age/parity. The relationship of maternal nutrition to prenatal fetal loss and neonatal survival and growth were studied in pigs that normally experience 40% loss of the fetus (Hard and Anderson, 1979). Sows from the first conception show growth over 3-4 parities to double their size (120-150 kg of body weight) and range of about 280-300 kg of body weight as adults. Maternal anabolism is greater in young than in older sows, because they have a higher growth path of the mother as adults (Young et al., 2005)

Figure 1 shows that porcine fetal growth accelerates during the second half of pregnancy (Knight et al., 1977; Wu et al., 1999; Pond and Mersmann, 2001). For example, Ulrey et al. (1965) already showed that the rate of growth of pigs fetal tissues with various stages of gestation. However, changes in composition of the fetus tissues during pregnancy have not been characterized. The availability of such data is essential to calculate the nutrient demand for growth of fetal tissue at a specific stage of gestation. Nutritional requirements for fetal tissue growth have impact on the overall maternal nutrition (NRC, 1998). In particular, maternal nutrients in the diet are mainly oriented to support the growth of fetal tissues (Trottier and Johnston, 2001). When the nutrient intake of the mother is insufficient, especially for pregnant sows, their growth, longevity and reproductive efficiency is reduced. Therefore, proper nutrition for pregnant gilts is important not only for growth and fetal development, but also to sow reproductive performance and lactation (Schoknecht, 1997; Kim and Easter, 2003). Therefore, the nutritional requirements for pregnant sows should be based on dynamic changes in the composition in different tissues of the fetus during gestation, in order to establish an effective strategy for feeding pregnant sows.

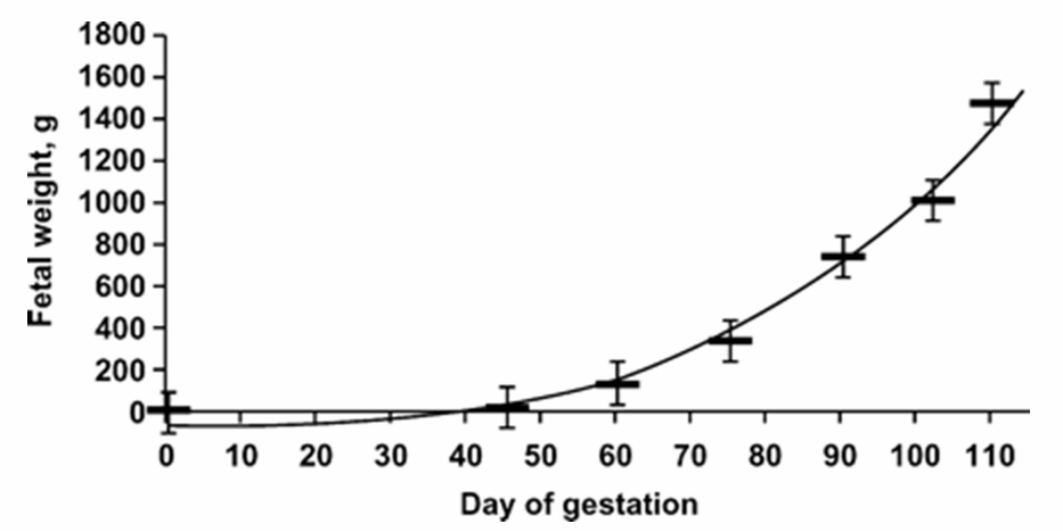


Figure 1: Porcine fetal growth (where y = fetal weight (g) and x = day of gestation) (Source: McPherson et al., 2004)

Knowledge about nutritional requirements is principally important for pregnant sows because their intake is usually restricted, and nutrients (minerals, vitamins and fiber) will be shortly considered, and total protein requirements are not constant throughout gestation. During the 1^{st} month of pregnancy, when conceptus growth accelerates, a change in both the priority and the demand for nutrients appear. In the first two months of pregnancy, most of nutrients are accumulated in maternal tissues (Noblet et al, 1997; Whittemore and Morgan, 1990). Then, at a fixed nutrient intake, the increase in requirements for conceptus gain is achieved at the expense of tissue deposition within the maternal body.

In particular, protein requirement of pregnant sows have been provided and information on the requirements of gestating sows is given by the official standards such as ITP (1991), NRC (1998) and FEDNA (2006) and have been studied and reviewed by several authors such as Pettigrew (1995), Nobert et al. (1997), Close and Cole (2003). Proteins and amino acids are usually calculated using the factorial approach, as the sum of the requirements for maintenance, maternal weight gain and products of conception. Speer (1990) and McPherson et al. (2004) reported that the accumulation of protein in the fetus increased from day 70 of gestation until the last month of gestation, which it is a particularly

important period. During pregnancy, protein requirements can separate into requirements for maintenance, accumulation of reproductive tissue and in particular conceptus tissue, and the gain of the mother. According to fetal growth, protein and amino acids requirements also increase with increasing gestation and become higher during the last month of pregnancy (Noblet and Etienne, 1987, Evert and Dekker, 1994, Dourmad et al., 1996).

The ideal protein of diets for gestating sows is changeable because the amount of amino acids conserved in various parts is different and difficult to measure (Cerisuelo, 2007, Table 1).

Table 1: Balance of essential amino acids in ideal protein of diets for gestating sows (lysin=1.00).

Amino acid	Maintenance	Conceptus	Maternal body	Total requirements
Lysine	100	100	100	100
Methionine	25	24	29	37
Cystine	111	22	15	-
Threonine	147	56	53	71
Tryptophan	30	12	12	20
Isoleucine	44	49	55	70

Source: Adapted from Cerisuelo (2007)

Among the amino acids lysine is usually the first limiting essential amino acids in the diet commercially available. An important element that affects lysine requirement during pregnancy is the gain of the mother and composition. Requirements for lysine are higher in pregnancy, when the maximum fetal development occurs, but also in gilts, in which the growth of the mother is more important than in adult sows. In practical terms, the balance of essential amino acids recommended for pregnant sows is the combination of maintenance, useful in maternal and reproductive functions.

Table 2: Mineral and vitamin requirements and recommended allowances for pregnant sows (% or amount/kg of diet) according to three different sources (NRC, 1998; BSAS, 2003 and FEDNA, 2006).

	NRC (1998)[1]	BSAS (2003)[2]	FEDNA (2006)[2]
Mineral			
Calcium, %	0.75	0.72	0.85-1.10
Digestible phosphorus (min), %	0.35	0.23	0.28
Sodium (min), %	0.15	0.17	0.18
Chloride (min), %	0.12	0.14	0.16
Zinc, ppm	50	80	100
Iron, ppm	80	80	75
Selenium, ppm	0.15	0.2	0.3
Vitamin			
Vitamin A, I.U.	4000	8500	10500
Vitamin D3, I.U.	200	800	1600
Vitamin E, I.U.	44	50	45
Vitamin K (menadione, ppm)	0.50	1.5	1.6
Thiamin (B1, ppm)	1.00	2	1.6
Riboflavin (B2, ppm)	3.75	5	5
Nicotinic acid, ppm	-	20	-
Pantothenic acid, ppm	12	15	13
Pyridoxyne (B6, ppm)	1.00	3	2.5
Cyanocobalamin (B12, ppb)	0.015	0.03	0.025
Biotin, ppb	200	200	130
Folic acid, ppm	1.30	3	2.1
Choline, ppm	125	300	260

Source: Nutient Requirement Council, 1998 (NRC); British Society for Animal Sciences, 2003 (BSAS); Fundacion Espanola para el Desarrollo de la Nutricion Animal, 2006 (FEDNA)

[1]Requirements [2]Recommended levels, amount of vitamins to be added per kg final compounded air-dry feed

For fetal development to precede normally, high concentrations of specific nutrients are often necessary during certain periods of pregnancy. An example would be the case of minerals and vitamins, as they are needed in small quantities but are essential for normal metabolism and animal health. Table 2 shows that the need for minerals and vitamins for pregnant sows established from different sources (NRC, 1998; BSAS, 2003 and FEDNA, 2006). Calcium and phosphorus and some trace elements such as sodium, chloride, zinc, iron and selenium are involved in various biological functions such as the proper development of the skeleton, the osmotic regulation of body fluids and other metabolic processes.

2.2 Substrates and co-factors of the one-carbon metabolism

The biological functions of components and co-factors of the one-carbon metabolism are briefly described in the following sections. Many micronutrients and vitamins are essential for DNA synthesis/maintenance and repair of DNA methylation. The one-carbon cycle delivers methyl groups that are covalently bound to cytosine by DNA methyl transferases resulting in different level of DNA-methylation (Figure 2). DNA methylation is a prominent mechanism of epigenetic regulation of gene expression. DNA methylation is a substantial molecular equivalent of the metabolic memory of nutritional (fetal) programming. Several substrats and co-factors are involved in the one-carbon cycle. These were considered when formulating the experimental diet in the present study.

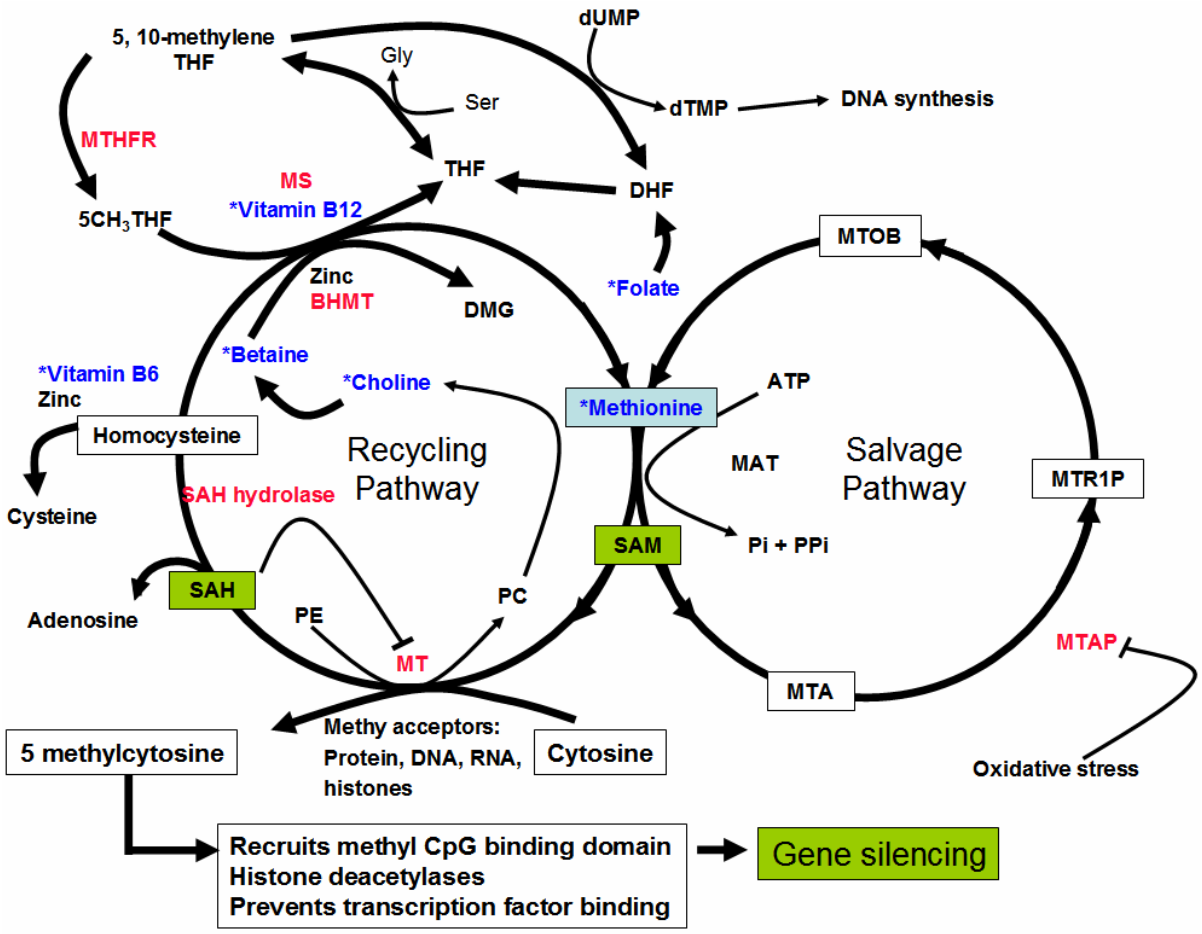


Figure 2: Metabolism of methionine and homocysteine.Schematic of the metabolic interrelations of methionine, choline, zinc, betaine, vitamine B12, vitamine B6, and folate.

SAH, S-adenosylhomocysteine
MTA, Methylthioadenosine
SAM, S-adenosylmethionine
MS, Methionine Synthase
MTHFR, Methylenetetrahydrofolate reductase
5CH3THF, 5-methytetrahydrofolate
THF, Tetrahydrofolate
MAT, Methionine adenosyltransferase
MTOB, 4-methylthio-2-oxobutanoic acid
MTAP, Methylthioadenosine Phosphorylase
monophosphate
PC, Phosphatidylcholine
Gly, Glycine
BHMT, Betaine methyltransferase
MTR1P, Methythioribose-1-PO4
PE, Phosphathidylethanolamine
PC, Phosphatidylcholine
MT, methyltransferase
Ser, Serine
DMG, Dimethylglycine
dTMP, deoxythymidine
dUMP, deoxyuridine monophosphate

Methionine

Methionine, which is not only essential for protein synthesis but is also involved in methylation reactions (Ball et al., 2006; Baker, 2006) are critical for DNA methylation and choline metabolism (Niculescu and Zeisel, 2002). Methionine is the precursor of homocysteine, a sulfur amino acid intermediate in the methylation and transsulfuration pathway. Excess dietary methionine drives a transient increase in methionine, S-adenosylmethionine (SAM), and homocysteine (Clayton et al., 2005). The methyl groups needed for all biological methylation reaction are derived from dietary methyl donors and from cofactors carrying one-carbon units. Substrates for SAM methyl transfer reactions include DNA, RNA, proteins, neurotransmitters and phospholipids (Troen et al., 2003).

Changes of the methylation of DNA comprise one of the major epigenetic modifications of the genome and play a significant role in the in the control of gene expression. Nutritional supply may affect early growth and development through the influence on gene expression, either by DNA methylation (covalent addition of a methyl group to cytosines in CpG dinucleotides) (Reik et al., 2001) and/or chromatin packaging of DNA via posttranslational modification of histones (Li, 2002; Turek-Plewa and Jagodziński, 2005) and the modification and assembly of regulatory protein complexes on DNA (Mann and Bartolomei, 2002; Cezar, 2003). These modifications can be functionally linked and are reversible.

Choline

Choline is an essential nutrient that functions in multiple physiological pathways that can be broadly divided into 3 categories: 1) as a component of phosphatidylcholine and sphingomyelin, which are critically important in cell membrane lipids, lung surfactant, bile lipids, and plasma lipoproteins. 2) As a part of the neurotransmitter acetylcholine. 3) as an important factor in methyl metabolism as a source of one-carbon groups for remethylation of homocysteine to methionine and for the folate pool (Friesen et al., 2007). Choline, or its metabolites, are needed for the structural integrity and signaling functions of cell membranes; it is the major source of methyl-groups in the diet (one of choline's metabolites, betaine, participates in the methylation of homocysteine to form methionine),

and it directly affects cholinergic neurotransmission, transmembrane signaling and lipid transport/metabolism (Zeisel, 2006; Zeisel and Blusztajn, 1994).

Betain

Betaine is obtained in small amounts from food or is generated from choline. It is also important because of it serves as a methyl donor in a reaction converting homocysteine to methionine, catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT). Betaine is an important animal nutrient obtained from the diet from a variety of foods. It is rapidly absorbed and utilized as an osmolyte and a source of methyl groups and thereby helps to maintain liver, heart, and kidney health. Betaine can reduce the elevated serum homocysteine concentrations associated with mild or severe hyperhomocystinuria via the methionine cycle and may play a role in epigenetics and athletic performance (Craig, 2004). The principal physiologic role of betaine is as an osmolyte and a methyl donor (transmethylation). As an osmolyte, betaine protects cells, proteins, and enzymes from environmental stress (eg, low water, high salinity, or extreme temperature). As a methyl donor, betaine participates in the methionine cycle primarily in the human liver and kidneys. This pathway is confined to liver and kidney and represents an alternative route of homocysteine remethylation, a reaction that is also performed by the ubiquitous folate-dependent methionine synthase (Finkelstein et al., 1972; Millian and Garrow, 1998). Inadequate dietary intake of methyl groups leads to hypomethylation in many important pathways, disturbed hepatic protein (methionine) metabolism as determined by elevated plasma homocysteine concentrations and decreased *S*-adenosylmethionine concentrations, inadequate hepatic fat metabolism, which leads to steatosis (fatty accumulation) and subsequent plasma dyslipidemia. This alteration in liver metabolism may contribute to various diseases, including coronary, cerebral, hepatic, and vascular diseases. Betaine has been shown to protect internal organs, improve vascular risk factors, and enhance performance. Databases of betaine content in food are being developed for correlation with population health studies. The growing body of evidence shows that betaine is an important nutrient for the prevention of chronic disease.

Folate

Folic acid has been more broadly studied in the context of nutritional programming because of its unique function as methyl donor for the synthesis of nucleotides and methylation of DNA. Cell culture and animal and human studies have shown that folic acid deficiency causes breaks in DNA and changes in the state of methylation of DNA. Animal models of methyl deficiency demonstrated an even stronger effect, not only in studies with a diet deficient in folate (Friesen et al., 2007). There are negative effects of inadequate folate status on the metabolism of DNA that may be mainly due to insufficient supply of methyl. It has recently been shown an interaction between folate status and a common mutation of the gene for methylenetetrahydrofolate reductase, an enzyme important in the metabolism of one-carbon. This finding suggests that the interaction between a nutritional status and genetic polymorphisms can modulate the regulation of gene expression on the level of DNA methylation.

Vitamin B6 and B12

Vitamin B6 (Pyridoxin) and B12 (deoxyadenosyl- or methyl-colbalamin) are important water-soluble vitamins belonging to the vitamin B complex. Vit B6 acts as a co-factor in the reaction catalysed by BHMT (betaine methyltransferase). Together with folic acid Vit B12 functions as methyl donor in the synthesis of DNA and in red blood cells as well as in maintaining the health of the insulation sheath (myelin sheath) that surrounds nerve cells. Vitamin B12-dependent methionine synthase is an important enzyme for sulphur amino acid, folate polyamine metabolism, S-adenosylmethionine metabolism and also in the methylation pathway of DNA, RNA, proteins and lipids (Banerjee and Matthews, 1990; Kenyon et al. 1999). The mammalian forms of methionine synthase require cobalamin as a prosthetic factor, *S*-adenosylmethionine (SAM) as a cofactor and a reducing system for activity (Banerjee and Matthews, 1990)

2.3 DNA methylation and interactions between nutrients and gene expression

DNA methylation, which is both genome and gene-specific, is of particular interest in research on cancer, aging and other conditions related to cell cycle regulation and differentiation of tissue-specific, because it affects gene expression without permanent

alterations in DNA sequence, such as mutations or allele deletions. The understanding of patterns of DNA methylation through interaction with nutrients is essential not only to provide pathophysiological explanations for the onset of disease, but also enhance the knowledge of possible strategies for prevention, changing the nutritional status of vulnerable populations (Niculescu and Zeisel, 2002). DNA-methylation is one of the main mechanisms of nutritional programming.

DNA methylation plays an important role in transcriptional regulation, chromatin remodeling and genomic stability. It is catalyzed in mammalian cells by a family of highly related DNA methyltransferases (DNMTs) that use S-adenosylmethionine as the methyl donor (Russell and Betts, 2008). DNA methylation at cytosine residues is a heritable epigenetic modification of the genome that dictates the reorganization of the chromatin packaging into a repressed state (Jaenisch and Bird, 2003). While DNA methylation patterns remain fairly unaltered in adult cells, they undergo dynamic modifications during early embryonic development when waves of demethylation and de novo methylation of the genome allow the transformation of inherited epigenetic patterns into embryo specific patterns (Razin and Shemer, 1995; Jaenisch, 1997) (Figure 2).

Epigenetic marks such as DNA methylation and histone modifications influence the binding affinity of *trans*-acting transcription activators/repressors and recruit chromatin remodeling enzymes, thus changing the structure and function of chromatin locally. These modifications play important roles in biological processes such as X inactivation, genomic imprinting, and transposon silencing. As epigenetic modifications might be stably inherited through mitotic division, without changes in DNA sequence, epigenetic information can be seen as a mechanism for cellular memory (Branco et al., 2008). The field of gene-nutrient interactions and DNA methylation, a fundamental epigenetic feature of DNA that affects gene expression and genomic integrity, is of growing interest. Several nutrients are involved in the maintenance of DNA metabolism, however most convincing data indicate a critical role for folate, an essential vitamin for DNA metabolism because it is involved in both DNA synthesis/repair and DNA methylation. The observation of an interaction between a common mutation in MTHFR (methylentetrahydrofolat-reductase), a key

enzyme of the one-carbon metabolic pathway, and DNA methylation provides the basis for research on the potential role of nutrients in modulating an epigenetic feature of DNA as well as in possible future prevention strategies (Figure 2).

2.4 Breed differences

Using the pig model, we aim at demonstrating foetal programming in pigs, listing genes sensitive to foetal programming, and evidencing and quantifying the role of DNA-methylation in this phenomenon. In order to address the impact that the genetic background may have on response to modified nutritional supply in terms of gene expression and DNA methylation animals of two breeds are used that show substantial phenotypic differences. The breed Deutsch Landrace was started about the year 1900 in northwest Germany and especially in Lower Saxony. Deutsch Landrace appears much like the Landrace of other nations, especially those from which they imported stock. They have been selected for meatiness and are not as extreme in size and length as some of the Landrace strains of other nations. The color is white and the ears are heavy with the characteristic Landrace droop. Selection efforts have been especially directed toward excellent fertility, heavy milking and good mothering characteristics. There has been special attention following weaning, to efficiency in feed conversion and to high cut-out values. Deutsch Landrace has proven very useful in crossbreeding and in improving the general performance of swine in Germany. The breed Pietrain became popular in its native country (Belgium) and was exported to other countries, especially Germany. The breed is of medium size and is white with black spots. They are shorter of leg than most breeds, stockier in build, and quite broad along the back. The hams are extremely bulging and muscular. They carry an extremely high proportion of lean to fat. When compared to the Landrace which is itself known for its meatiness, they excelled in usable lean. These comparative figures and its reputation for very high quality lean, makes the breed a desired one for fresh meat processing.

The study focuses on liver, because it plays a central role in the regulation of the metabolic status, the partitioning of nutrients and the expenditure of energy. Ultrastructural studies of pig fetal livers show that its development can be divided into three periods: a period of differentiation [18–40 days postconception (dpc)], a period of metabolic activity (40–80 dpc), and a period of glycogen accumulation (80–113 dpc) (Bielańska-Osuchowska, 1996).

Prenatal hepatic expression profiles revealed functional networks that reflect the stage-specific major hepatic activities and indicated, that Pietrain tend to exhibit higher expression of genes involved in changes and development of tissues and cells whereas Deutsch Landrace tend to show higher transcript abundance of genes involved in intracellular energy and metabolite conversion. These trends are even visible at prenatal stages of development that are targets of nutritional programming (Ponsuksili et al., 2007). The insulin axis is involved in the development of a number of different tissues including liver, muscle and adipose tissue. These tissues learn to communicate with each other during the late fetal and early postnatal stages of development. The development of liver, muscle and adipose tissue is regulated by gene nutrient interactions. If the nutritional balance is perturbed by changes in the maternal diet, the development of tissues is also changed altering the characteristics of the feedback loop of the insulin axis. The communication between these organs is a complex web where subtle changes in just one component can influence the behavior of the whole system. (Maloney and Rees, 2005)

2.5 Application of microarray in farm animal

Microarrays are useful tools to study affect of various factors such as genetics (Lehnert et al., 2006), nutrition level (Ollier et al., 2007), type of diet (Bernabucci et al. 2004), animal management (Cogburn et al., 2004) and treatments on gene expression in tissues or cells (Hocquette et al., 2005), especially for revealing novel genes that have not previously been involved in a physiological or nutritional response. To conduct microarray experiments on animals for breeding a number of commercial arrays (Table 3) and customer arrays are usable and commercially available. There are also numerous vendors offering the production of application-specific arrays based on sequence information from customers.

Table 3: Selected commercial available microarray resources for farm animals (source: Bendixen et al., 2005)

Name	Array type	No. spots/probe	No. genes	Vendor[c]
GeneChip® Porcine Genome Array	High density oligo	23937	20201	Affymetrix
GeneChip®Chicken Genome Array[a]	High density oligo		28000	Affymetrix
GeneChip® Bovine Genome Array	High density oligo	24072	23000	Affymetrix
Pig Genome Oligo Set ver. 1.0	Array ready oligos	10665	10665	Operon
Pig Genome Oligo Extension Set ver. 1.0	Array ready oligos	2632	2632	Operon
Pig Immune Array	cDNA microarray		2860	ARK genomics
Chicken Embryo Array	cDNA microarray		1152	ARK genomics
Chicken Immune Array	cDNA microarray		5000	ARK genomics
Chicken Neuroendocrine Array	cDNA microarray		4800	ARK genomics
Pig Oligo array[b]	Oligo microarray	20400	20400	ARK genomics
Porcine Gene Expression Microarray	Oligo microarray	43803		Agilent

[a] This array also contains 689 probe sets for detecting 684 transcripts from 17 avian viruses.

[b] Qiagen Pig Genome Oligo Set Version 1.0 and the Pig Genome Oligo Extension Set Version 1.0 printed on one slide.

[c] Affymetrix, Operon, ARK Genomics, Agilent.

Microarrays containing characterized genes are available for domestic animals such as cows, pigs and chickens. Microarray studies in farm animals were done in regard of: gene expression related to muscle growth potential (ovine, bovine) and between normal and double muscled bovines (Hamelin et al., 2006; Cassar-Malek et al., 2007), breed-specific expression of bovine liver and intestine in view of genes involved in processing of nutrients in cattle (Schwerin et al., 2006), between different feeding regiments, and different cattle breeds (Byrne et al., 2005; Wang et al., 2005; Lehnert et al., 2006), aetiology of fatty liver in dairy cows at calving (Bernabucci et al. 2004), response of genes to nutritional or physiological status in the intestine (Shirazi-Beechey, 2004), genes expressed in placenta

and uterine tissues between non-pregnant and pregnant cows. (Hocquette et al., 2005), increased capacity for gluconeogenesis in early lactation in cows (Greenfield et al. 2000), muscle of ruminants (Hocquette et al. 2001), adipose tissues (Bonnet et al. 2000), in mammary gland and liver between non-lactating and lactating cows (Hocquette et al., 2005; Loor et al., 2006) and beef sensory quality (Bernard et al., 2007; Lehnert et al., 2007; Morzel et al., 2008). There are some reports in lamb and chicken. For example, Diez-Tascon et al. (2005) detected genes differentially expressed between lambs being genetically resistant or susceptible to larval nematodes. In mammary gland genes differentially expressed depending on the feeding level in goats, that were detected (Ollier et al., 2007). Cogburn et al. (2004) used microarrays to study differential gene expression in the liver of embryos and hatching chicks, and between fasting and refed chickens.

In swine, Ponsuksili et al. (2008) studied genes differentially expressed in muscle that affect water-holding capacity of pork and hepatic gene expression profiles in pre- and postnatal of DL and PI pigs differing in body composition (Ponsuksili et al., 2007), in muscles between breeds and muscle types, and between carriers of the RN-mutations and wild-type pigs (Mullen et al., 2006; Tuggle et al., 2007). The construction of cDNA microarrays from normalized porcine muscle was reported by Yao et al. (2002) and from brain cDNA libraries by Nobis et al. (2003). Yao et al. (2004) performed experiments to identify genes expressed in foetal ovary and Bai et al. (2003) used porcine cDNA microarrays containing 5500 clones to studied differential transcript expression in muscle for identifying the genes associated in muscle phenotype. The annotation of the Affymetrix porcine genome microarray was constructed by Tsai et al. (2006). Naraballobh et al. (2010) reported an updated annotation and in silico localization of the Affymetrix GeneChip Porcine Genome Array probe sets based on build 9 of the porcine genome sequence (Ensembl_Sscrofa_9).

3. Materials and methods

3.1 Materials

3.1.1 Animals and Experimental Design

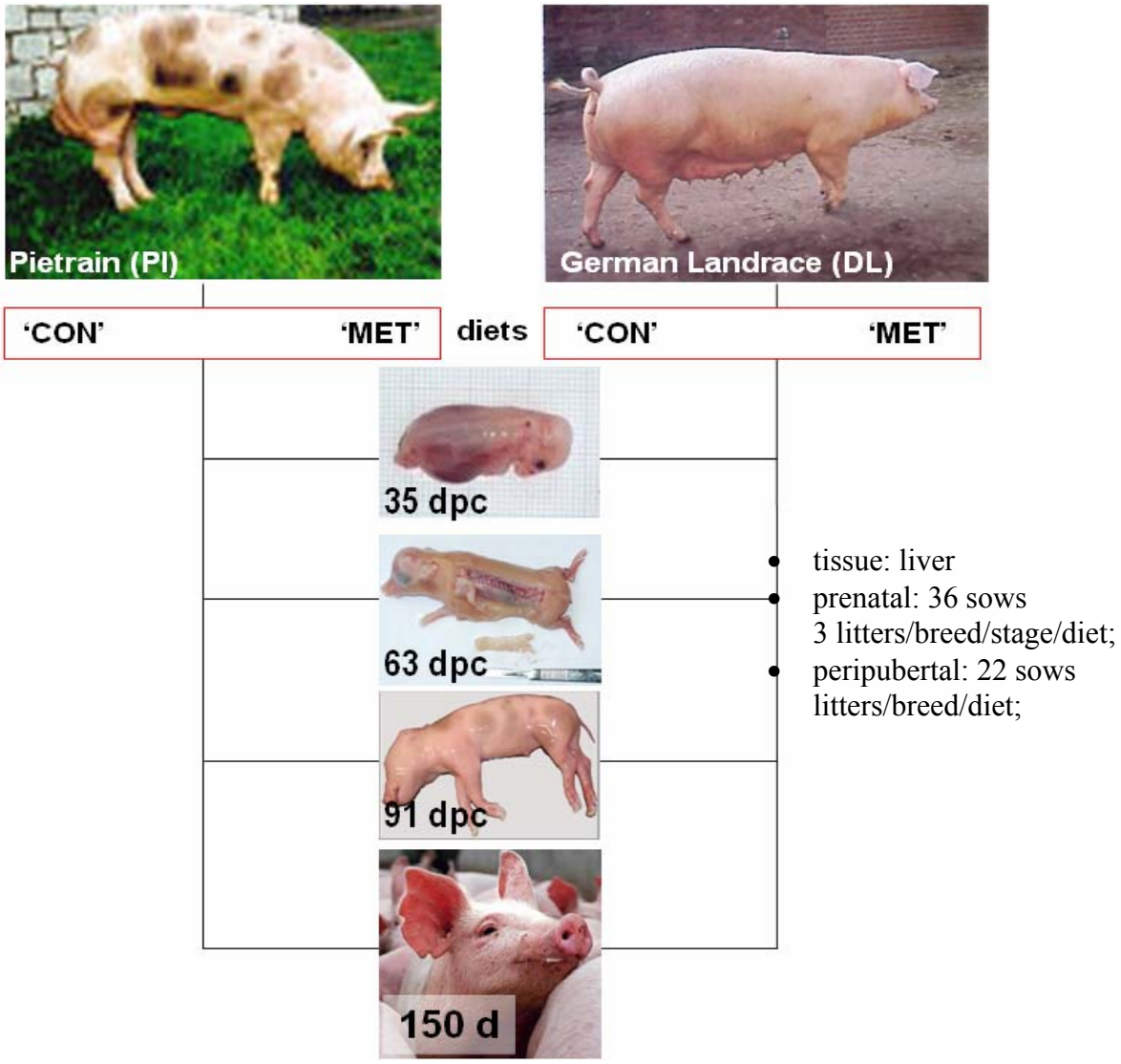


Figure 3: The diagram shows experimental design and the timing of sampling

The whole experiment used 59 sows of the breeds German Landrace (DL) and Pietrain (Pi) that were fed methionine supplemented (`MET´) vs. control (`CON´) gestation diets that were different in 6 components: Zinc, Vitamin B6, Vitamin B12, Folate, Choline and Methionine (Table 4). Expression profiling of liver was done of samples of a total of six sows, foetuses of three developmental stages (35, 63, 91 dpc (post conceptionem)), and offspring at peripubtertal age (d 150 p.n. (postnatum)). Liver samples of fetuses were

subsequently monitored for diet-dependent differential gene expression by using Affymetrix micorarrays, total number of hybridizations show in Table 6.

Table 4: Composition of standard gestation diet and experimental diets (as fed)

	Unit	Declaration	
		`CON´ diet	`MET´ diet
Trace elements / Heavy metals			
Zinc	mg/kg	21.8	149
Vitamin			
Vitamin B6	mg/kg	3	1180
Vitamin B12	ug/kg	31	5930
Folate	mg/kg	3	92.2
Choline	mg/kg	500	2230
Amino acids			
Methionine	mg/kg	2050	4700

Therefore, 36 sows were fed methionine supplemented (`MET´) vs. control (`CON´) gestation diets. In total 79, 81, and 74 fetuses at the respective stages (35, 63, 91 dpc) were obtained to build up repositories of liver and muscle tissues (M18_2.5a). Liver samples of fetuses of three developmental stages (35, 63, 91 dpc) were subsequently monitored for diet-dependent differential gene expression by using Affymetrix micorarrays (3 sows (=litters) per breed, per stage, per diet; 2 pools of 6 animals each per litter; total of 72 hybridisations).

23 sows of both breeds (DL n=13, PI n=10) were fed the control and experimental gestation diets in order to obtain samples of their offspring at peripubertal age. For the breed DL and PI 96 and 56 offspring were obtained. The offspring were born, raised at the research farm of the FBN Dummerstorf and the Faculty of Agricultural and Environmental Sciences of the University of Rostock, and performance tested at the performance test station Jurgensdorf of the Hybrid pig breeders federation North/East (Hybridschweinezuchtverband Nord/Ost e.V.).

Table 5: Overview of traits recorded

Trait category	Trait	Trait description
growth performance	bw_d7	body weight at 7 days of life
	bw_d14	body weight at 14 days of life
	bw_d21	body weight at 21 days of life
	bw_abs	body weight at weaning (28 days of life)
	bw_w12	body weight at 12 weeks of life
	bw_w18	body weight at 18 weeks of life
	bw_sla	body weight at slaughter (average 158 days of life)
carcass traits	cw_hot_le	carcass weight warm, left half
	cw_hot_ri	carcass weight warm, right half
	cw_hot	carcass weight warm
	cw_cold_le	carcass weight cold, left half
	cl	carcass length
	ham_lenght	ham length
	ham_size	ham circumference
	mar	marbling score 1 lean to 5 fat
	belly score	belly score 1 lean to 9 fat
	bf_neck	backfat measured at neck
	bf_13th/14th	backfat measured at at $13^{th}/14^{th}$ rib
	bf_lumbal	backfat measured at last lumbar
	meat%	meat percentage
meat quality traits	pH1mld	pH-value in M. longissimus dorsi (mld) at $13^{th}/14^{th}$ rib 1 h post mortem
	pH24mld	pH-value in M. longissimus dorsi (mld) at $13^{th}/14^{th}$ rib 24 h post mortem
	pH1msm	pH-value in M. semimembranosus (msm) at 1 h post mortem
	pH24msm	pH-value in M. semimembranosus (msm) at 24 h post mortem
	cond1mld	conductivity in Mld at $13^{th}/14^{th}$ rib 1 h post mortem
	cond24mld	conductivity in Mld at $13^{th}/14^{th}$ rib 24 h post mortem
	DRIP	Drip loss: percentage of weight loss after 48 h of Mld samples collected at
	OPTO	meat colour 24 h post mortem in *M. longissimus dorsi* (Mld)at $13^{th}/14^{th}$
	Minolta L	meat colour 24 h post mortem in *M. longissimus dorsi* (Mld)at $13^{th}/14^{th}$
	Minolta a	meat colour 24 h post mortem in *M. longissimus dorsi* (Mld)at $13^{th}/14^{th}$
	Minolta b	meat colour 24 h post mortem in *M. longissimus dorsi* (Mld)at $13^{th}/14^{th}$
	IMF	intramuscular fat of
	water	% water in Mld
	protein	% protein in Mld
	ashes	% ashes in Mld

Traits related to growth performance were recorded (Table 5). The pigs were slaughtered at the abattoir of the FBN-Dummerstorf and carcass and meat quality data were collected according to guidelines of the Zentralverband der Deutschen Schweineproduktion e.V. (ZDS, 2004). Animals were slaughtered at a mean age of 158 days.

Data were analysed using the Proc `Mixed´ of SAS statistic software package applying mixed models taking into account for the random effect of mother and the fixed effects of gestation diet, gender, breed, RYR1 genotype, batch. Weight at slaughter and age at slaughter were considered for carcass and meat quality traits. For ordinal/nominal traits Proc `glimmix´ and Proq `freq´ were used

3.1.2 Equipment

Vortex	G-560E	Scientific Industries Inc., USA
Centrifuge	5417C	Eppendorf-Netheler-Hinz GmbH, Hamburg
Centrifuge	Z320	Hermle-Labortechnik, Wehingen
Heater	FDB02AD	Techne (Cambridge) Ltd., England
UV transilluminator	TFML-40	LTF-Labortechnik GmbH & CO. KG, Wasserburg
PCR thermocycler	HBPX 110	Thermo Electron GmbH, Ulm
Spectrophotometer	ND-1000	Nanodrop technologies, USA
Power supply	GFS200/400	Phamacia, Sweden
Electrophoresis box	-	Angewandte Gentechnologische Systeme GmbH, Heidelberg

3.1.3 Buffers, Chemicals, reagents and kits

dNTPs	Promega, Mannheim, Germany
Buffer (10X)	Sigma-Aldrich, Taufkirchen, Germany
Oligonucleotide primer	Sigma-Aldrich, Taufkirchen, Germany
Taq polymerase	Sigma-Aldrich, Taufkirchen, Germany
Agarose	Sigma-Aldrich, Taufkirchen, Germany

TAE (50X)	Tris 2 M
	Acetic acid 57% (v/v)
	EDTA, pH8.0 0.05 M
PCR purification kit	QIAGEN, Hilden, Germany
DnaseI	Roche, Mannheim, Germany
TRIzol reagent	Sigma-Aldrich, Taufkirchen, Germany
Qiagen Rneasy kit	QIAGEN, Hilden, Germany
Phenol/Chloroform (50:50)	Sigma-Aldrich, Taufkirchen, Germany
Chloroform	Sigma-Aldrich, Taufkirchen, Germany
Gibco BRL®	Life Technologies, Karlsruhe, Germany

3.1.4 Software

Primer3 v. 0.4.0	http://frodo.wi.mit.edu/primer3
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
SAS version 9.3	SAS Institute Inc., USA
JMP Genomics version 3.2	SAS Institute, Cary, NC
ArrayAsist package	Stratagene
Ingenuity Pathway Analysis Software	Ingenuity Systems, USA
Expression console	Affymetrix, Santa Clara, CA
QVALUE software	Storey and Tibshirani

Other specific equipment, buffers, chemical reagents and kits, as well as software are mentioned in the following text.

3.2 Methods

3.2.1 Tissue collection

Animals were exsanguinated and slaughtered as described above (Figure 3). Tissue samples of the fetus livers were collected following the timing diagram shows in Figure 3, immediately frozen in liquid nitrogen and stored at -80 °C until isolation of RNA.

3.2.2 RNA isolation

We collected liver samples of fetuses of three postnatal stages (35, 63, 91 dpc) and postnatal stage which were subsequently scanned for diet-dependent differential gene expression by using Affymetrix GeneChips Porcine Genome Arrays (Affymetrix, Inc., Santa Clara, CA). As a first step frozen tissues of liver were ground with a mortar in liquid nitrogen to avoid unfreezing and degradation of RNA, 1 ml of TRIZOL reagent was added and homogenized by using syringes. The homogenized samples were incubated for 5 min at room temperature. Then 0.2 ml of chloroform was added and mixed thoroughly by shaking and incubated for 15 min at 15 – 30 °C. Samples were centrifuged at 12,000 g for 15 min at 2 to 8 °C. The RNA remains exclusively in the upper aqueous phase which was transferred to a fresh tube. Add 0.5 ml of phenol/chloroform and centrifuged at 12,000 g for 5 min at 4 °C. Then transfer aqueous phase to a new then add 0.5 ml of chloroform and centrifuged at 12,000 g for 5 min at 4 °C. The RNA in the aqueous phase was precipitated by adding 0.5 ml of isopropyl alcohol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 2 to 8 °C. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75 % ethanol. After centrifugation and removal of supernatant the RNA pellet was air dried and dissolved in 50 µl RNase-free water and stored at –80 °C for further use. To remove residual of DNA, the RNA was treated with DNase and incubated at room temperature for 30 minutes. After incubation, the RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentration and purity of the RNA were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies Ltd, USA) by measuring the optical density of each sample at 230, 260 and 280 nm. The 260/280 and 260/230 (protein-contamination-value) ratios should be above 1.8 to ensure high purity of RNA. The quality of the RNA was further confirmed by gel electrophoresis on 1% gel. On the gel, the bands of the 18S

and 28S rRNA band should be clearly visible. To control fragment sizes, the Alu I Marker was used.

3.2.3 RNA Pooled

RNA pooled of prenatal stage from DL and PI was prepared (Table 6). Individual RNA samples of postnatal stage were used of individual animals from DL and PI in `CON´ and `MET´.

Table 6: Overview of microarray hybridisations.

Treatment	Breed	Stage	Sows	Microarray chip	Animal
`CON´	DL	35	2268	KDL35-2268-1	
			2268	KDL35-2268-2	
			2279	KDL35-2279-1	2 pools of 6 samples per sow
			2279	KDL35-2279-2	
			2282	KDL35-2282-1	
			2282	KDL35-2282-2	
		63	2027	KDL63-2027-1	
			2027	KDL63-2027-2	
			2273	KDL63-2273-1	2 pools of 6 samples per sow
			2273	KDL63-2273-2	
			2281	KDL63-2281-1	
			2281	KDL63-2281-2	
		91	2006	KDL91-2006-1	
			2006	KDL91-2006-2	2 pools of 6 samples per sow
			2015	KDL91-2015-1	
			2026	KDL91-2026-1	
			2026	KDL91-2026-2	
		Postnatal	2490	KDLpost-45023	L45023
			2490	KDLpost-45037	L45037
			2490	KDLpost-45044	L45044
			2671	KDLpost-45116	L45116
			2671	KDLpost-45129	L45129
			2671	KDLpost-45134	L45134

Table 6: (Continue) Overview of microarray hybridisations.

Treatment	Breed	Stage	Sows	Microarray chip	Animal
`CON´	PI	35	6081	KPI35-6081-1	2 pools of 6 samples per sow
			6081	KPI35-6081-2	
			6282	KPI35-6282-1	
			6282	KPI35-6282-2	
			6139	KPI35-6139-1	
		63	6146	KPI63-6146-1	2 pools of 6 samples per sow
			6146	KPI63-6146-2	
			6152	KPI63-6152-1	
			6152	KPI63-6152-2	
			6324	KPI63-6324-1	
			6324	KPI63-6324-2	
		91	6270	KPI91-6270-1	2 pools of 6 samples per sow
			6270	KPI91-6270-2	
			6327	KPI91-6327-1	
			6327	KPI91-6327-2	
			6331	KPI91-6331-1	
			6331	KPI91-6331-2	
		Postnatal	201	KDLpost-55007	L55007
			105	KDLpost-55015	L55015
			1503	KDLpost-55053	L55053
			1304	KDLpost-55065	L55065
			807	KDLpost-55104	L55104
`MET´	DL	35	2375	VDL35-2375-1	2 pools of 6 samples per sow
			2375	VDL35-2375-2	
			2376	VDL35-2376-1	
			2376	VDL35-2376-2	
			2369	KDL35-2369-1	
		63	2276	VDL63-2276-1	2 pools of 6 samples per sow
			2276	VDL63-2276-2	
			2002	VDL63-2002-1	
			2002	VDL63-2002-2	
			2280	VDL63-2280-1	
			2280	VDL63-2280-2	
		91	2005	VDL91-2005-1	2 pools of 6 samples per sow
			2005	VDL91-2005-2	
			2007	VDL91-2007-1	
			2007	VDL91-2007-2	
			2016	VDL91-2016-1	
			2016	VDL91-2016-2	
		Postnatal	2477	VDLpost-45019	L45019
			2485	VDLpost-45033	L45033
			2477	VDLpost-45040	L45040
			2672	VDLpost-45112	L45112
			2672	VDLpost-45127	L45127
			2672	VDLpost-45136	L45136

Table 6: (Continue) Overview of microarray hybridisations.

Treatment	Breed	Stage	Sows	Microarray chip	Animal
`MET´	PI	35	6279	VPI35-6279-1	2 pools of 6 samples per sow
			6279	VPI35-6279-2	
			6323	VPI35-6323-1	
			6323	VPI35-6323-2	
			6154	VPI35-6154-1	
		63	6264	VPI63-6264-1	2 pools of 6 samples per sow
			6264	VPI63-6264-2	
			6283	VPI63-6283-1	
			6283	VPI63-6283-2	
			6325	VPI63-6325-1	
			6325	VPI63-6325-2	
		91	6153	VPI91-6153-1	2 pools of 6 samples per sow
			6328	VPI91-6328-1	
			6328	VPI91-6328-2	
			6332	VPI91-6332-1	
			6332	VPI91-6332-2	
		Postnatal	403	VDLpost-55004	L55004
			405	VDLpost-55018	L55018
			1103	VDLpost-55048	L55048
			1603	VDLpost-55061	L55061
			1608	VDLpost-55070	L55070

Microarray chips used to perform analysis highlighted by *shading.*

3.2.4 Affymetrix hybridization

67 pools of RNA from fetuses and 22 individual RNA from postnatal stage were reversely transcribed into cDNA and added to the in vitro transcription reaction to generate biotinylated cRNA. The biotinylated cRNA was hybridized to 89 Affymetrix GeneChips Porcine Genome Arrays (Affymetrix, Inc., Santa Clara, CA). Microarrays were washed with Fluidics Station 450 (Affymetrix, Inc., Santa Clara, CA) and scanned with GeneChip® Scanner 3000 (Affymetrix, Inc., Santa Clara, CA). Data was analyzed with the Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500.

3.2.5 Expression analysis and Statistical analysis

The quality of hybridization was assessed in all samples by using ArrayAssist Expression Software (Stratagene Corp, CA, USA). 78 microarray chips used to perform analysis highlighted by shading (Table 6). Expression values were calculated using the Gene Chips Robust Multi-array Average (GC-RMA) method and signal values were transformed to log 2 and statistical analysis was performed to detect genes differentially regulated at $p < 0.05$

due to the gestation diet using Row-by-Row Modeling (mixed model analysis) within breed and stage, taking in to account the fixed effects of `diet´ and `mother´ nested within`diet´.

$$Y = \mu + diet + mother\ (diet)$$

A set of corresponding *q-values* was calculated using Row-by-Row Modeling (P-value adjustment) of JMP Genomics Software version 3.2 (SAS Institute Inc., North Carolina, USA). We selected list of differentially expressed genes using a threshold of fold change in gene expression > 1.5. In the following text the differentially expression genes are termed DE genes; however also the term gene, transcript, or probe set is used ion order to refer to differences of expression patterns.

3.2.6 Ingenuity pathway analysis

The Ingenuity Pathway Analysis system (Ingenuity Systems Inc, Redwood City, CA) was used to analyze the functional networks and canonical pathways associated with the significant differentially expressed genes. The annotation of affymetrix probe sets to gene was taking from Naraballobh et al. (2010). The core and comparison analyses for canonical pathways were used to analyze up-down regulation data and corresponding fold changes data. Briefly, for a given canonical pathway, statistical significance of pathway enrichment is calculated using a Fisher's exact test with a significance level of 0.05 and modified option of customize chart on canonical pathway by selecting metabolic pathways and signaling pathways (apoptosis, cell cycle regulation, cell growth, proliferation and development, cellular stress and injury, growth factor signaling, intracellular and second messenger signaling, nuclear receptor signaling, organismal growth and development)

3.2.7 Quantitative real-time RT-PCR

Differential expression data of selected genes obtained from Porcine Genome Arrays were validated by using quantitative real-time PCR on a LightCycler ® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using LightCycler DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The primers were derived from Affymetrix core sequences of the corresponding probe sets (http://dripdb.molgen.mpg.de) and designed using Primer3, which is available at http://frodo.wi.mit.edu (Table 7 and Table 8). Total RNA from individual animals were reversely transcribed, then subjected to quatitative real-time PCR analysis. The levels of a given mRNA were normalized against the RPL32 mRNA level.

cDNA synthesis were performed with 5 mg of total RNA using 100 ng of random hexamers and SuperScriptIII Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. Expression levels were normalized to RPL32 gene expression, which was used as an internal housekeeping control. PCR mixtures (final volume of 12 µl) contained 6.0 ul of the LightCycler 480 PCR Master SYBR Green (Roche Diagnostics), 2 µl of a 1:10 dilution of the cDNA, 2.8 µl water (Roche Diagnostics), and 0.6 µl (10 mM) of each primer (Table 7 and Table 8). The cycling conditions included an initial heat-denaturing step at 95°C for 5 min, 40 cycles at 95°C for 20 s, annealing at 60°C for 15 s for each of the primers, and product elongation at 72°C for 15 s and signal acquisition (single mode) at 72°C for 10 s.

Following amplification, the melting curves were determined in a three-segment cycle of 95°C for 5 s, 60°C for 1 min, and 95°C for 0 S at the continuous acquisition mode. The temperature transition rates were set at 20°C/s except for segment three of the melting curve analysis where it was set to 0.1°C/s. The amplification was conducted in duplicate according to manufacturer's instructions using 10 µM of each primer. 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). At the completion of the amplification protocol, all samples were subjected to melting curve analyses and gel electrophoresis to verify the absence of any non-specific product. To

account for variation in RNA input and efficiency of reverse transcription the calculated mRNA copy numbers were normalized by dividing with a normalization factor derived from the expression of the reference gene. In total, 24 individual liver mRNA samples were analyzed in duplicate per stage. Data were analyzed using the PROC MIXED, including effects of `diet´and `mother´ nest within `diet´ (SAS version 9.1; SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$.

Table 7: List of the primers used for the quantitative real-time PCR in DL.

Probe ID set	Gene name	Sequence 5'→3'	AT[b][°C]	Length of PCR product [bp]	FT[c][°C]
Ssc.16105.1.S1_at	BHMT	For - TCCGTTTTCGAGTACCATCC Rev - CACATAGCCCCTCTTCTCCA	60	150	72
Ssc.28076.1.A1_at	DNMT1	For - TCAGGGACCACATCTGTAAGG Rev - GCTGCAGCCATTCTTCTTGT	60	177	72
Ssc.7298.1.A1_at	AMD1	For - TTTACCGGTTTTGCTAAGAAGC Rev - GGACTTTCTGCAACTATAAGGTGG	60	167	72
Ssc.15588.1.S1_at	IGFBP3	For - ACAAGGTCGACTACGAGT Rev - GTAGAAGCCCTTCTTGTC	60	182	72
Ssc.15800.1.S1_at	IGFBP5	For - TACTCGCCCAAGATCTTC Rev - TGCTCAGATTCCTGTCTC	60	173	72
Ssc.5093.1.A1_at	SHC1	For - CGGTAGCGTTTCTACATCACC Rev - GTGGAAGGCCCAAGTTTCTC	60	168	72
Ssc.30159.1.A1_at	AAK1	For - ATGCAGCGTCATTTGTTAGG Rev - CATGCACATATTGTTTTCTGAGGT	60	175	72
Ssc.7262.1.A1_at	SMAD5	For - CAATGAGGCCAGACAATCC Rev - AAAATGCAACTTTAACCAACCA	60	174	72
Ssc.10311.2.A1_at	MAP3K7	For - CATGCAGTTCACCTGTCTCC Rev - CACATAGCAGCTAGTTTGATACCC	60	164	72
Ssc.16231.2.A1_a_at	IGF1	For - ACTCGTGCAGAGCAAAGGAT Rev - CCCCCATCTACCAACAAGAA	60	169	72
Ssc.2827.1.S1_at	SOCS3	For - AGACCCCGGACAGAGAAGAT Rev - CTGGATTGGCTGAGTGTGT	60	134	72
	RPL32	For - AGCCCAAGATCGTCAAAAG Rev - TGTTGCTCCCATAACCAATG	60		72

Table 8: List of the primers used for the quantitative real-time PCR in PI.

Probe ID set	Gene name	Sequence 5'→3'	AT[b][°C]	Length of PCR product [bp]	FT[c][°C]
Ssc.835.1.S1_at	PFN	For - CTGATGGGCAAAGAAGGTGT Rev - GATGTGTGTGTGTGGGAAGG	60	154	72
Ssc.4127.2.A1_at	Rho	For - TGGCAGGAGAGATGATTAAGG Rev - AGCACTTGCCAGCTATTTTGT	60	178	72
Ssc.2878.1.A1_at	PAK	For - GCACCTGTACACATCAGTCATAAA Rev - ACATGCAGGTAACCATCAAGGAA	60	150	72
Ssc.15588.1.S1_at	IGFBP3	For - ACAAGGTCGACTACGAGT Rev - GTAGAAGCCCTTCTTGTC	60	182	72
Ssc.15800.1.S1_at	IGFBP5	For - TACTCGCCCAAGATCTTC Rev - TGCTCAGATTCCTGTCTC	60	173	72
Ssc.422.1.S1_at	IGF2R	For - TTCCAGCTTGGGGTGTAGG Rev - GCAACGCTAGGGGTGAGA	60	190	72
Ssc.16105.1.S1_at	BHMT	For - TCCGTTTTCGAGTACCATCC Rev - CACATAGCCCCTCTTCTCCA	60	150	72
Ssc.28076.1.A1_at	DNMT1	For - TCAGGGACCACATCTGTAAGG Rev - GCTGCAGCCATTCTTCTTGT	60	177	72
Ssc.27049.1.A1_at	MTAP	For - ATATTTAATGGACTAAAGAAATGGTTG Rev - CCCCCAAATACAATAACAAAAG	60	160	72
Ssc.24383.1.S1_at	FOXO1	For - TATATCCCAGGGAGCAGGAA Rev - ATTTCCAGTTGGGGGTTAGG	60	171	72
Ssc.6811.2.S1_at	BCL2	For - CTTCTTCGGCATCAGAAAGC Rev - CCACACTGCTCCGTTTCATA	60	161	72
Ssc.4721.2.S1_at	MAFG	For - TTTGGTGAAAGGAACCAACC Rev - GACACACAGGCCAGAGCA	60	169	72
	RPL32	For - AGCCCAAGATCGTCAAAAG Rev - TGTTGCTCCCATAACCAATG	60		72

3.2.8 SAM and SAH measurements

To determine SAM and SAH content for calculation of the ratio SAM/SAH in liver tissue from fetuses and sows, liver tissue from 17 sows of DL and 18 sows of PI and pools of fetal liver representing `CON´ and `MET´ and all prenatal stages from DL (15 pools) and PI (13 pools) were used (Table 9). Therefore tissues were frozen at -80 °C and pulverized in liquid nitrogen, protein was precipitated in ice-cold of 0.6 N perchloric acid, and tissue adenosine levels were determined by using HPLC analysis, according to protocol from Kloor et al (2000). I would like to thank Dr. Doris Kloor (Department of Experimental and Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Tübingen, Germany) for running HPLC analysis.

Table 9: Number of fetuses and sows for measurement of SAM and SAH content.

Breed	Animal	35 dpc		63 dpc		93 dpc	
		`CON´	`MET´	`CON´	`MET´	`CON´	`MET´
DL	Sows	3 animals	2 animals	3 animals	3 animals	3 animals	3 animals
	Fetus	1 pools	1 pools	2 pools	2 pools	1 pools	2 pools
PI	Sows	3 animals	3 animals	3 animals	3 animals	3 animals	3 animals
	Fetus	1 pools	1 pools	2 pools	1 pools	2 pools	1 pools

3.2.9 Global DNA methylation

DNA samples from 3 stages of DL fetuses and PI fetuses (Table 10) were isolated for determination of global DNA methylation assessed by using the MethylampTM Global DNA Methylation Quantification Ultra Kit (Epigentek Group Inc., Brooklyn, NY, USA) according to the protocol provided by the manufacturer. Levels of DNA methylation were to the expressed relative to the Lsmean estimated for `CON´ over all stages and both breeed that were set 100.

Table 10: Number of fetuses for global DNA methylation determines.

Breed	Stage		
	35 dpc	63 dpc	93 dpc
DL	74 animals	77 animals	79 animals
PI	63 animals	63 animals	57 animals

4. Results

4.1 Phenotypes of offspring

The weight of the fetus in DL `CON´ and `MET´ diet group increased as gestation progressed. The weight of fetal carcasses in `MET´on 35 dpc was significantly lower than that in `CON´ (*P<0.001*) (Table 11), whereas the weight difference was not significant at 63, 91 dpc and postnatal stage. Carcasses weight of PI fetus in `MET´ on 35, 91 dpc and postnatal stage was significantly higher than in `CON´ (*P<0.05*) (Table 11), whereas the weight difference was not significant at 63 dpc.

Table 11: Carcasses weight on 35, 63, 91 day of postconception and postnatal stage in `CON´ and `MET´ diet.

Breed	Day of gestation	`CON´ diet			`MET´ diet.		
		Lsmean (g)	std err	animal	Lsmean (g)	std err	animal
DL	35 dpc*	4.30	0.06	45	3.40	0.04	31
	63 dpc	165.60	26.87	38	156.70	23.90	43
	91 dpc	684.40	119.15	33	643.20	101.71	40
	Postnatal	115100.00	106.00	30	114700.00	216.00	26
PI	35 dpc*	3.70	0.46	38	4.10	0.48	24
	63 dpc	145.70	25.710	33	134.60	21.99	30
	91 dpc*	574.40	162.40	29	690.10	127.30	27
	Postnatal*	98400.00	250.00	28	89600.00	210.00	26

* Significant differences P<0.05

The traits covered provide insight into the animal's growth performance, body and carcass composition and meat quality traits. According to the statistical evaluation gestation diets had significant effects only on traits related to meat colour; in particular lightness and yellowness of meat was affected by the diets fed to the mothers of the animals (Table 12).

Table 12: Results of analysis of variance for trait records obtained in both breeds

	`CON´		`MET´		DL `CON´		PI `CON´		DL `MET´		PI `MET´		diet	diet * breed	slice `diet´ within `breed´	diet * gender
	lsmean	std err	lsmean	std err	lsmean	std err	lsmean	std err	lsmean	std err	lsmean	std err				
bw_1	1.81	0.09	1.79	0.08	1.41	0.09	2.21	0.15	1.54	0.10	2.03	0.14	~	~		~
bw_28	7.83	0.44	7.46	0.41	8.25	0.60	7.41	0.68	8.38	0.63	6.54	0.65	~	~		~
bw_152	101.75	3.20	101.07	3.65	108.56	3.34	94.94	5.91	106.05	3.51	96.08	5.88	~	~		~
cw_hot_le	41.93	2.40	40.42	1.81	41.65	1.99	42.20	3.82	40.99	1.99	39.85	2.81	~	~		~
cw_hot_ri	40.57	2.27	39.14	1.72	40.47	1.89	40.67	3.62	40.01	1.88	38.26	2.67	~	~		~
cw_hot	80.96	3.68	80.70	3.73	84.98	3.19	76.95	6.84	85.10	3.39	76.30	6.26	~	~		~
cw_cold_le	40.52	2.43	39.92	1.82	39.50	2.04	41.55	3.86	40.45	2.04	39.40	2.83	~	~		~
bf_lumbar	1.53	0.15	1.57	0.13	1.69	0.12	1.38	0.27	1.73	0.13	1.41	0.23	~	~		~
meat%	58.93	0.80	58.56	0.68	56.84	0.61	61.02	1.47	55.98	0.63	61.14	1.23	~	~		~
pH1mld	6.47	0.04	6.43	0.04	6.53	0.03	6.41	0.08	6.49	0.03	6.38	0.07	~	~		~
pH24mld	5.46	0.02	5.44	0.02	5.45	0.01	5.46	0.04	5.46	0.01	5.43	0.04	~	~		~
cond1mld	4.41	0.17	4.49	0.16	4.04	0.14	4.78	0.31	4.14	0.14	4.83	0.30	~	~		~
cond24mld	4.20	0.18	4.34	0.16	3.39	0.11	5.02	0.33	3.50	0.12	5.18	0.30	~	~		~
OPTO	68.91	1.44	72.33	1.31	69.25	1.17	68.58	2.63	73.08	1.23	71.57	2.37	0.05	~	DL 0.046	~
DRIP	3.48	0.36	3.73	0.34	3.07	0.26	3.89	0.67	3.33	0.29	4.12	0.61	~	~		~
Minolta L	49.25	0.65	47.88	0.60	48.96	0.48	49.54	1.20	47.58	0.52	48.17	1.10	0.07	~	DL 0.065	~
Minolta a	6.82	0.18	7.01	0.16	6.97	0.12	6.66	0.34	7.13	0.13	6.89	0.30	~	~		~
Minolta b	1.57	0.19	1.28	0.17	1.54	0.12	1.60	0.35	1.09	0.14	1.47	0.31	0.11	*	DL 0.024	~
water	74.99	0.15	75.09	0.13	74.83	0.10	75.16	0.28	74.56	0.11	75.62	0.25	~	*	DL 0.093 PI 0.090	~
IMF	1.07	0.10	1.10	0.09	1.13	0.07	1.01	0.18	1.15	0.07	1.04	0.16	~	~		~

~ P> 0.1

Analyses of the data separate for each breed confirm significant effects of the gestation diets on meat colour in DL. The interaction `diet*breed´ also showed significant effect on meat colour. Interestingly, significant effects of `diet´ within the the level `DL´ of the effect of `breed´ were found (Table 12 and Table 13).

Table 13: Results of analysis of variance for trait records obtained in DL, (lsmeans ± standard errors)

Trait	DL `CON´		DL `MET´		effect `diet´ p-value
	lsmean	std err	lsmean	std err	
bw_1	1.41	0.05	1.53	0.05	
bw_28	7.77	0.46	7.9	0.5	
bw_152	107.92	2.38	107.16	2.44	
cw_hot_le	42.04	0.94	43.38	0.97	
cw_hot_ri	40.19	0.78	41.5	0.81	
cw_hot	84.66	2.15	85.39	2.19	
cw_cold_le	40.58	0.79	42.29	0.83	0.009
bf_lumbar	1.74	0.14	1.8	0.14	
meat%	56.7	0.71	55.81	0.72	
pH1mld	6.52	0.03	6.48	0.03	
pH24mld	5.45	0.01	5.46	0.02	
cond1mld	4.1	0.11	4.18	0.11	
cond24mld	3.45	0.1	3.53	0.11	
OPTO	68.94	1.15	72.92	1.19	0.048
DRIP	3.19	0.21	3.39	0.23	
Minolta L	49.07	0.46	47.68	0.49	0.065
Minolta a	6.98	0.11	7.12	0.12	
Minolta b	1.55	0.13	1.12	0.14	0.044
water	74.84	0.09	74.57	0.1	0.081
IMF	1.11	0.06	1.13	0.06	

Further there were differences in cold carcass weight in DL depending on the gestation diets. There are only subtle differences between the gestation diet groups within each breed. The nominal differences between gestation diet groups within breeds indicate a trend towards higher body weight before weaning and carcass weight in DL in `MET´, but in

`CON´ in PI (Table 14). Also for leanness and meat quality traits mostly opposite trends were observed in both breeds.

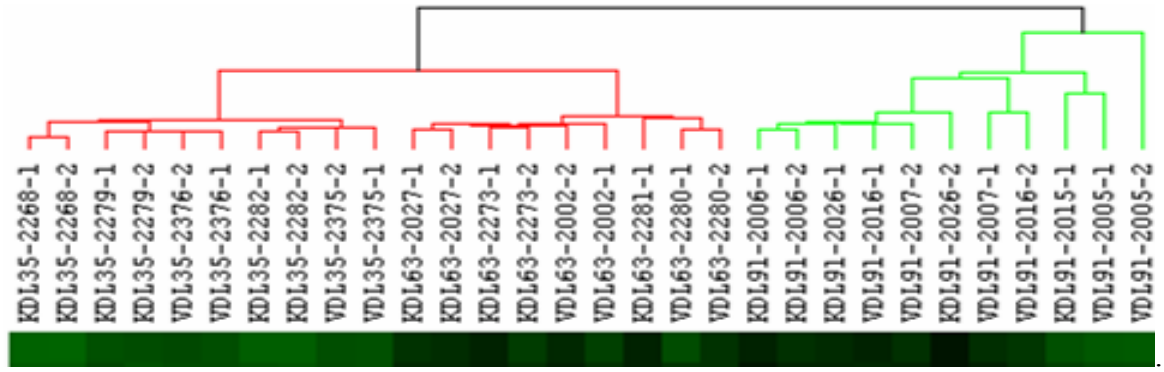
Table 14: Results of analysis of variance for trait records obtained in PI (lsmeans ± standard errors)

Trait	PI `CON´		PI `MET´	
	lsmean	std err	lsmean	std err
bw_1	2.13	0.16	1.83	0.17
bw_28	7.55	0.81	6.82	0.85
bw_152	100.4	3.84	97.34	5.16
cw_hot_le	40.56	3.55	38.58	3.55
cw_hot_ri	39.52	3.32	37.4	3.31
cw_hot	80.08	6.88	75.98	6.87
cw_cold_le	39.6	3.47	37.68	3.46
bf_lumbar	1.35	0.21	1.26	0.17
meat%	61.25	1.09	61.62	0.89
pH1mld	6.34	0.06	6.36	0.06
pH24mld	5.44	0.04	5.42	0.05
cond1mld	4.72	0.4	4.55	0.53
cond24mld	4.95	0.38	4.75	0.41
OPTO	67.43	0.289	69.89	3.34
DRIP	4.16	1.05	3.9	1.32
Minolta L	50.03	1.65	48.35	2.12
Minolta a	6.79	0.36	7.06	0.39
Minolta b	2.05	0.49	1.84	0.64
water	75.34	0.36	75.39	0.45
IMF	1.15	0.19	1.27	0.21

4.2 Expression analysis and statistical analysis

For the primary analysis we used ArrayAssist® Expression Software for checking of the expression data quality and hierarchicalclustering of expression profiles (Figure 4). Figure 4A shows expression profiles from DL that was separated into three distinct clusters: expression profiles data on 35 dpc formed one cluster, expression profiles on 63 dpc formed another, and expression profiles on 91 dpc formed a third cluster. Expression profiles data on 35 and 63 dpc clustered more tightly together than with chips of 91 dpc regardless of the analysis. These data show that clustering does not distinctly separated diet from `CON´ and `MET´ into each cluster of expression profiles data. After statistical analysis of expression data genes differentially expressed at $P<0.05$ (corresponding false discovery rate about 18.5%) were listed (Table 15). Hierarchical clustering in Figure 4B shows expression profiles from PI that were separated according to stage 35, 63 and 91 dpc. Statistical analysis was finished and selected genes at $P<0.05$ (corresponding false discovery rate about 70%) were listed (Table 15).

(A)



(B)

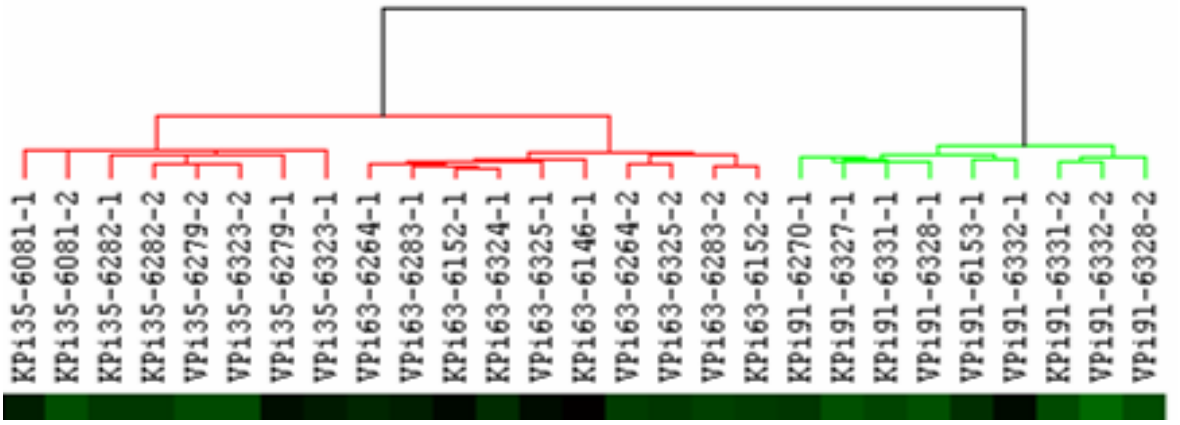


Figure 4: Hierarchical clustering of expression profiles of prenatal stages of `CON´ and `MET´ diet group in DL (A) and in PI (B).

By analysis of variance we obtained lists of genes differentially expressed due to gestation diets fed to the sows throughout pregnancy at thresholds of $p < 0.05$ and fold chance > 1.5 (Table 15). In order to perform bioinformatics analyses and biological interpretations of the data an up-to-date annotation of Affymetrix probe sets to EnsEMBL Sscofa 9 (20,439 of 23,935 annotated probe sets) was used (Naraballobh et al., 2010).

Table 15: Numerical summary of gene expression profiles and changes in DL and PI breed porcine liver between `CON´ vs `MET´ diet at different ontogenetic stages.

Breed	Stage (dpc)	Genes with Differentially Expressed ($p<0.05$) and fold changes > 1.5	q value	Annotated with Naraballobh et al. (2010)	Regulation	
					up	down
DL	35 dpc (10 chips)	2178	0 – 0.139	2055	128	1927
	63 dpc (9 chips)	703	0 – 0.184	641	191	450
	91 dpc (11 chips)	2678	0 – 0.233	2475	2337	138
	Postnatal (12 chips)	367	0 – 0.999	327	134	193
PI	35 dpc (8 chips)	639	0 - 0.887	618	23	595
	63 dpc (10 chips)	1650	0 - 0.622	1539	1526	13
	91 dpc (8 chips)	1669	0 - 0.613	1592	54	1538
	Postnatal (10 chips)	133	0 - 0.999	112	62	50

In DL, the hepatic expression profiles revealed 2055 transcripts that were differentially expressed at 35 dpc. At 63 dpc 641 transcripts were regulated due to maternal gestation diets. At these early prenatal stages the majority of probe sets were down regulated in `MET´ compared to `CON´. At 91 dpc the highest number of regulated probe sets was found with the majority being up-regulated. The expression value of 327 probe set IDs at postnatal stage were affected with 134 up- and 193 down-regulated probe sets in `MET´. For the prenatale stages q-values ranging from 0 to 0.23 were found; at postnatal stage it was up to 0.99.

In PI, at 35 dpc 618 annotated probe-sets were regulated, which is less than 1/3 of the number of probe-sets found to be regulated in DL at the same stage. More than 1500 differentially regulated genes were found at 63 dpc and at 91 dpc; at postnatal stage 112. In PI larger q-values were estimated than in DL (Table 15).

4.3 Genes overlapping among stages

Differential expression (DE) gene lists from DL obtained at three prenatal stages were analysed regarding those genes that showed consistent regulation at al three prenatal stages (35, 63 and 91 dpc). The common response to the gestation diets at the three stages was limited to 11 genes (LOC731957, SLCO3A1, NAV2, TTF1, TMEM47, STRBP, dJ421D16.1, E2F7, CDH5, ARL8B, VPS13D) as shown in Figure 5. The overlapping genes were involved in canonical pathways of TR/RXR activation, Gα12/13 signaling, hepatic cholestasis, Wnt/β-catenin signaling, leukocyte extravasation signaling.

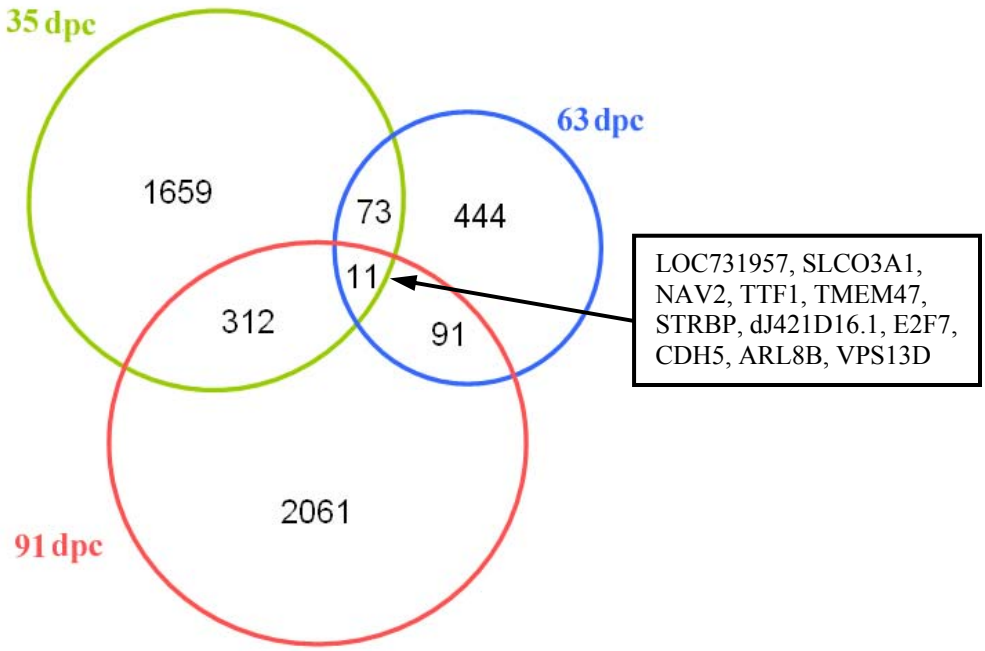


Figure 5: Venn diagram detailing the comparison of lists of differentially expressed probe-sets at three prenatal stages in DL. Numbers of regulated genes are given that are private to a particular stage and common to two or all three stages.

The overlapping of DE gene lists from PI among the three stages (35, 63 and 91 dpc). The common response to the gestation diets at the three stages was limited to 75 unique genes as shown in Figure 6. These gene are involved in 10 canonical pathways included sonic hedgehog signaling, aminoacyl-tRNA biosynthesis, bile acid biosynthesis, histidine metabolism, butanoate metabolism, valine leucine and isoleucine degradation, glycine serine and threonine metabolism, EIF2 signaling, IGF-1 signaling.

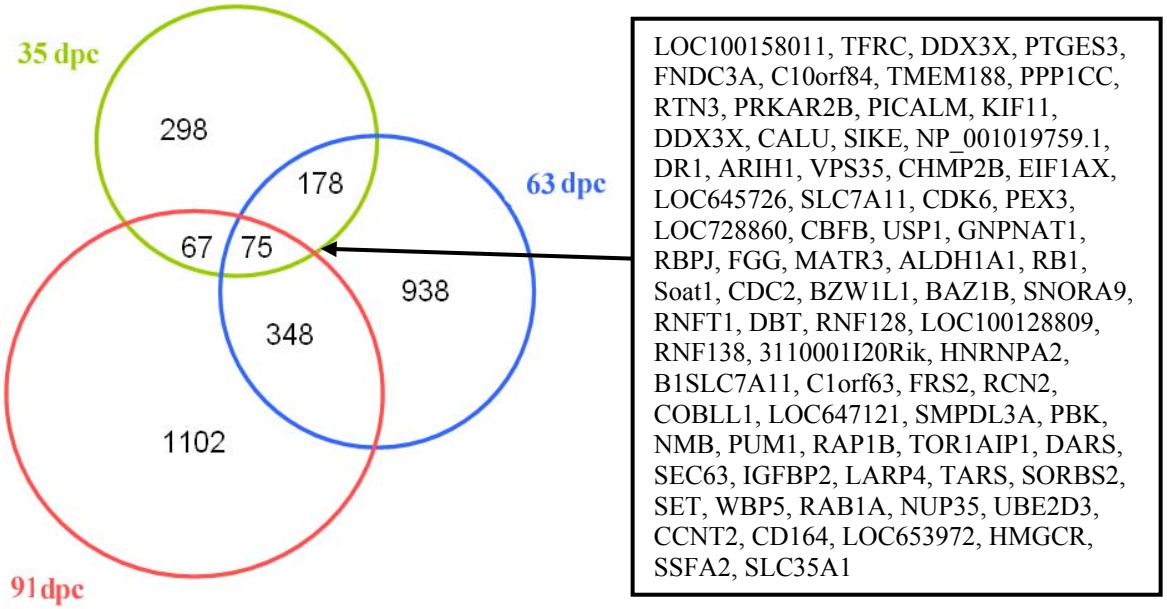


Figure 6: Venn diagram detailing the comparison of lists of differentially expressed probe-sets at three prenatal stages in PI. Numbers of regulated genes are given that are private to a particular stage and common to two or all three stages.

4.4 Identification of gene functions

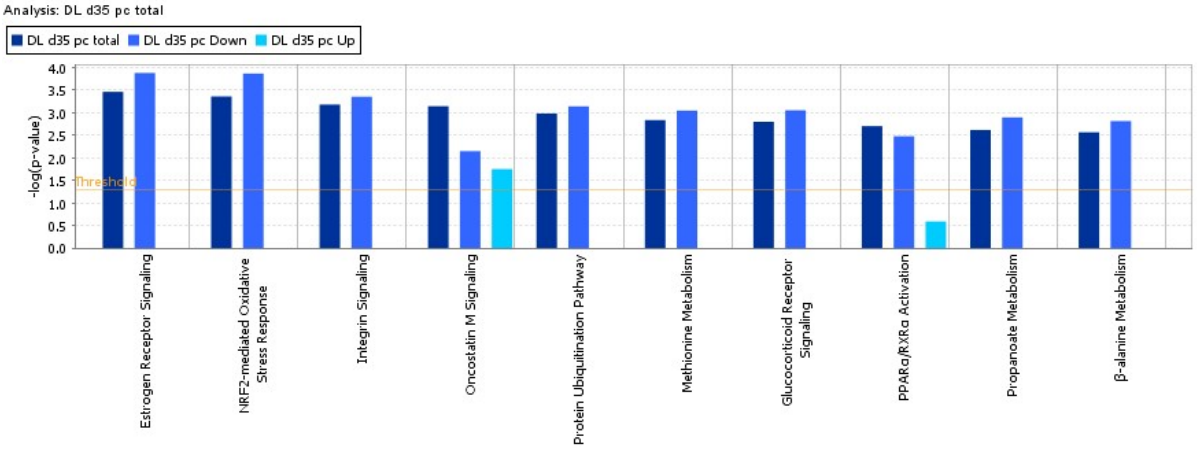
We used IPA pathway analysis software to investigate the biological relevance of the observed genome-wide changes by categorizing our data set into biological functions. Pathways were represented of the molecular relationships between genes and gene products. The intensity of a gene's (node) color in the networks indicates the degree of up-regulation (red) or down-regulation (green). Nodes are displayed using various shapes that represent the functional class of gene products.

4.4.1 IPA pathway analysis of gene functions in DL

Canonical pathways analysis revealed significant pathways that are affected at 35 dpc namely estrogen receptor signaling, NRF2-mediated oxidative stress response, integrin signaling, oncostatin M signaling, protein ubiquitination pathway, methionine metabolism, glucocorticoid receptor signaling, PPARα/RXRα activation, propanoate metabolism, β-alanine metabolism. This is detaled in Figure 7A, where the pathways known to be affected at 35 dpc in liver RNA between `CON´ and `MET´. Colors of bars represented 3 data sets, i.e. all genes regulated and genes up- or down-regulated in `MET´ compared to `CON´. The significance threshold at $p < 0.05$ of enrichment is shown as a yellow line. Bars that are above the line indicate significant enrichment of a pathway. The canonical pathways that exhibit a significant higher number of genes found to be regulated between `MET´ and `CON´ than expected by chance alone could be assigned to the functional categories of amino acid metabolism, cellular growth, proliferation development, intercellular and second messenger signaling, nuclear receptor signaling, carbohydrate metabolism, ingenuity toxicity list pathways (Table 16).

The methionine metabolism pathway at 35 dpc (Figure 7B) was among the important canonical pathway. There were 10 genes differentially expressed between the `CON´ and `MET´ (TRDMT1, SRM, AMD1, AHCYL1, BHMT, ALAS1, TAT, MTR, MAT2B, DNMT1) that are shown in Table 16. All of the involved genes were down-regulation in the `MET´. We selected AMD1, BHMT, DNMT1 from this pathway for validation of differential expression by real-time PCR.

(A)



(B)

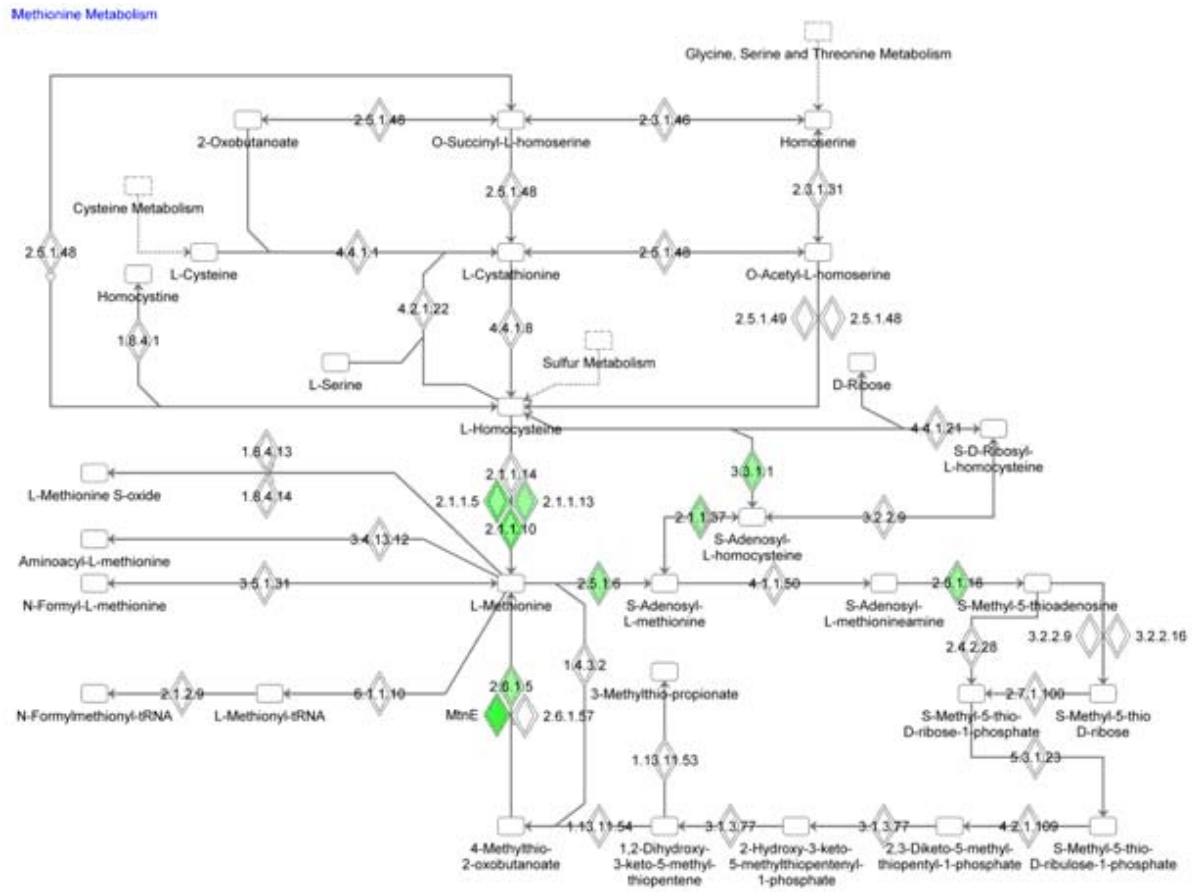


Figure 7: IPA canonical pathways that were significantly affected in DL at 35 dpc. (A) Diagram of IPA canonical pathway of methionine metabolism with enzymes encoded by genes down-regulated in `MET´ highlighted in green (B).

Table 16: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from DL at 35 dpc.

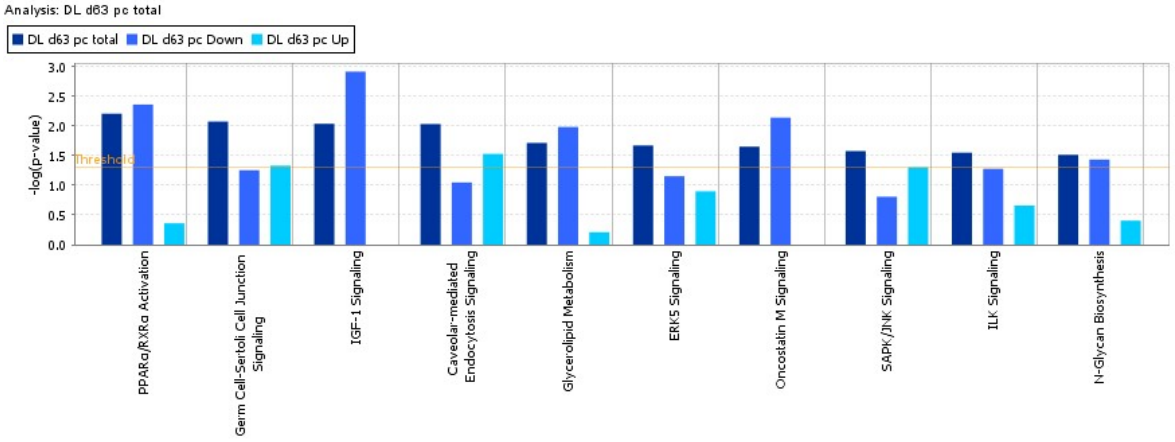
Categories	Ingenuity Canonical Pathways	-log(p-value)	Molecules
amino acid metabolism	methionine metabolism	2.83	TRDMT1, SRM, AMD1, AHCYL1, BHMT, ALAS1, TAT, MTR, MAT2B, DNMT1
	β-alanine metabolism	2.56	ALDH4A1, ALDH2, SRM, HADHB, ACADL, DPYS, ALDH1A1, ABAT, ACAD8, ECH1, ACADSB, ALDH7A1
cellular growth, proliferation development	integrin signaling	3.18	MAP2K4, MAPK1, CDC42, ARPC5, PPP1CB, LIMS1, PARVB, PAK1, ITGA9, ARF4, ARPC4, ITGAV, MRAS, RHOU, ILKAP, VCL, MAP2K1, VASP, ACTN1, PAK2, GRB2, ASAP1, CRKL, ITGA2, BCAR3, GIT1, ITGB3, ARF5, PAK3, CAPN2, ACTN4, RAP2A
	oncostatin M signaling	3.14	TIMP3, MAPK1, GRB2, MT2A, MRAS, MMP13, OSMR, STAT3, PLAU, MAP2K1
intercellular and second messenger signaling	protein ubiquitination pathway	2.98	USP14, USP12, UBE2A, UBE2N, UBE2L3, USP11, HSPA5, USP39, USP48, UBE4A, USP7, HLA-A, UCHL5, UBE2D3 (includes EG:7323), UBE2E3, ANAPC11, UBE2J1, AMFR, UBE2R2 (includes EG:54926), USP30, USP1, PSMD3, PSMD5, SKP1, SKP2, HSPA8, PSMD11, PSMA4, UBC, USP34, BIRC2
	glucocorticoid receptor signaling	2.80	MAP2K4, MAPK1, NFATC3, SMAD3, PBX1, SMARCD2, TAF7, TAF13, SLPI, HSPA5, NR3C1, FGG, TGFBR2, TSC22D3, HMGB1 (includes EG:3146), NCOA2, MRAS, FKBP5, SERPINE1, MAP2K1, TAF12 (includes EG:6883), MAP2K7, GRB2, MED1, TAF15, TAF5L, HSPA9, CREBBP, TBP, MAPK9, TAT, STAT3, MED14, HSPA8, KAT2B, MAPK14, TAF4, NFATC2
nuclear receptor signaling	estrogen receptor signaling	3.46	TAF12 (includes EG:6883), MAPK1, TAF15, GRB2, MED1, TAF5L, CREBBP, TBP, TAF7, TAF13, NR3C1, G6PC3, MED14, CTBP1, KAT2B, DDX5, HDAC3, TAF4, NCOA2, MRAS, MAP2K1, PPARGC1A
	PPARα/RXRα activation	2.70	MAP2K4, GPD1, NCOA6, MAPK1, PRKAB2, TGFBR3, SMAD3, GNA11, MAP4K4, ABCA1, TGFBR2, PLCD3, MRAS, MAP2K1, PRKCA, MAP2K7, MED1, GRB2, ACOX1, CREBBP, ADCY6, CAND1, ACADL, MAPK14, RXRA, PPARGC1A, PRKAR1A

Table 16: (Continue) Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from DL at 35 dpc.

Categories	Ingenuity Canonical Pathways	-log(p-value)	Molecules
carbohydrate metabolism	propanoate metabolism	2.61	ALDH4A1, SUCLG2, ABAT, ECH1, ACADSB, MUT, ALDH2, HADHB, SUCLA2, ACADL, ALDH1A1, ACAD8, ACSL1, ALDH7A1
ingenuity toxicity list pathways	NRF2-mediated oxidative stress response	3.36	MAP2K4, USP14, MAPK1, DNAJB4, GCLC, DNAJC3, DNAJA1, VCP, MRAS, DNAJC1, DNAJA2, FKBP5, MAP2K1, PRKCA, MAP2K7, DNAJC9, CREBBP, MAPK9, DNAJB9, TXNRD1, DNAJC11, MAPK14, MGST2, STIP1, CAT, MAPK7, PTPLAD1, DNAJB5, DNAJC7, MGST3

At 63 dpc canonical pathways analysis revealed pathways that were significantly affected, namely PPARα/RXRα activation, germ cell-sertoli cell junction signaling, IGF-1 signaling, caveolar-mediated endocytosis signaling, glycerolipid metabolism, ERK5 signaling, oncostatin M signaling, SAPK/JNK signaling, ILK signaling, N-glycan biosynthesis (Figure 8A). These canonical pathways belong to the biofunctions of growth Factor signaling, cellular growth, proliferation development, organismal growth and development, metabolism of coplex lipids, intercellular and second messenger signaling, nuclear receptor signaling, apoptosis, glycan synthesis and metabolism (Table 17). Figure 8B shows the IGF-1 signaling pathway, an important canonical pathway influenced at 63 dpc with 8 affected genes (SHC1, FOXO1, YWHAH, IRS1, IGFBP3, IGFBP5, MAP2K1, PRKAG1). The three genes were chosen for validation IGFBP3, IGFBP5 and SHC1.

(A)



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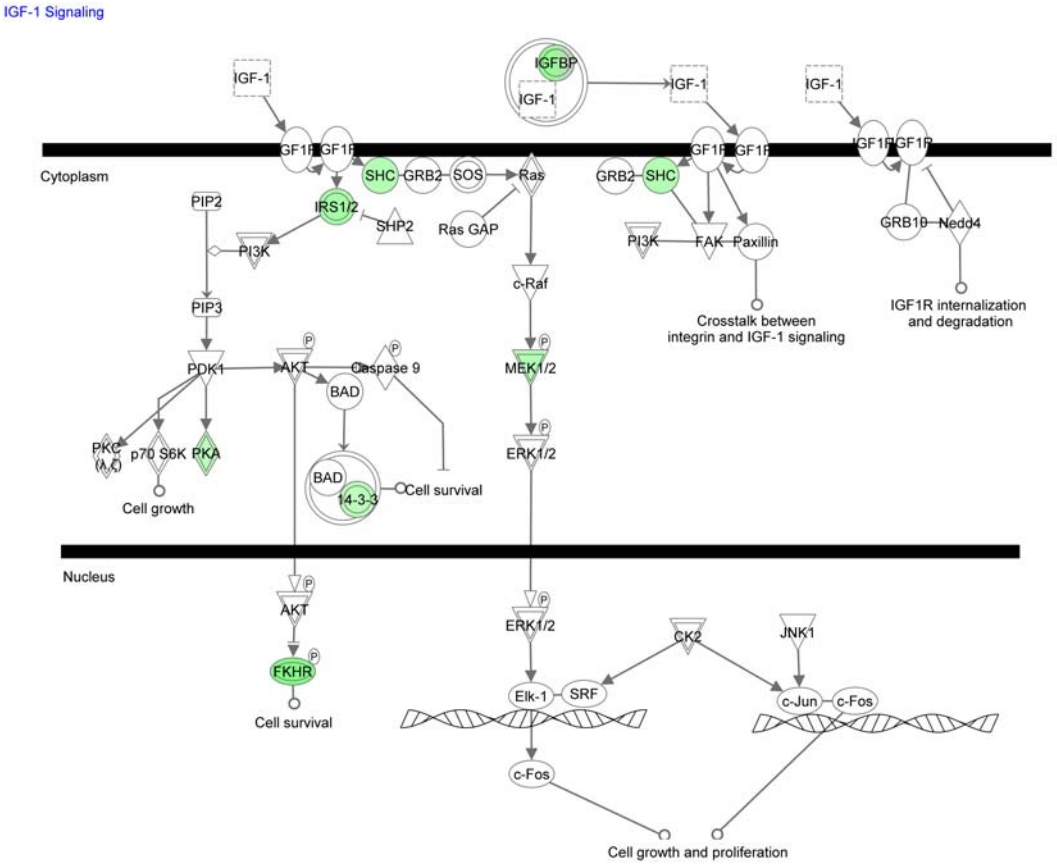
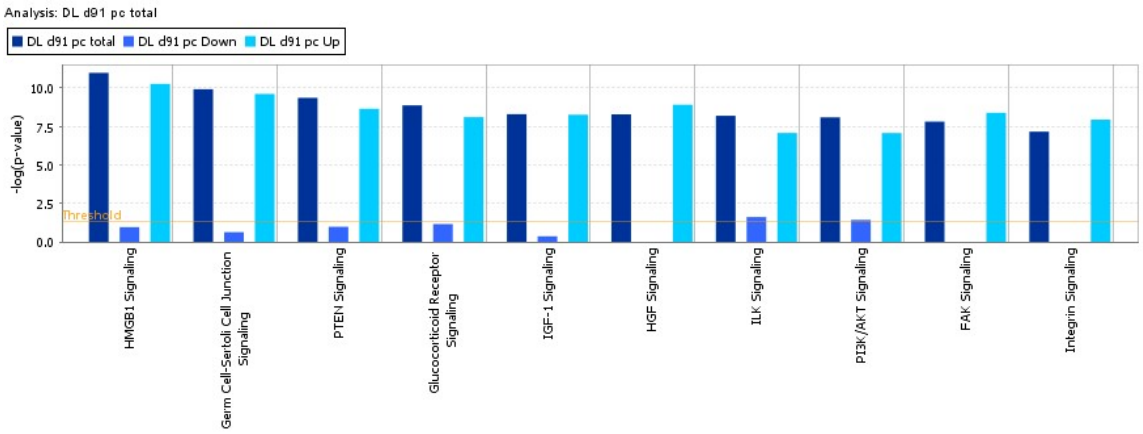


Figure 8: IPA canonical pathways that were significantly affected in DL at 63 dpc. (A) Diagram of IPA canonical pathway of IGF-1 signaling pathway with components encoded by genes down-regulated in `MET´ highlighted in green (B).

Table 17: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from DL at 63 dpc.

Categories	Ingenuity Canonical Pathways	-log (p-value)	Molecules
growth factor signaling	IGF-1 signaling	2.03	SHC1, FOXO1, YWHAH, IRS1, IGFBP3, IGFBP5, MAP2K1, PRKAG1
cellular growth, proliferation development	germ cell-sertoli cell sunction signaling	2.07	WASL (includes EG:8976), RND3, MAP3K7, RHOU, TGFB2, ACTG2 (includes EG:72), ACTC1, MAP2K1, MAP3K3, ACTA1, MAP3K2
	oncostatin M signaling	1.65	SHC1, MT2A, STAT5B, MAP2K1
	ILK signaling	1.54	NCK2, ITGB2, FBLIM1, RND3, IRS1, SH2B2, RHOU, RPS6KA5, ACTG2 (includes EG:72), ACTC1, ACTA1
organismal growth and development	caveolar-mediated endocytosis signaling	2.03	ITGB2, FLOT2, HLA-A, ACTG2 (includes EG:72), ACTC1, ACTA1, MAP3K2
metabolism of coplex lipids	glycerolipid metabolism	1.71	ALDH1B1, LIPA, DGAT2, DGKE, UGT2B10, LPIN2, AGPAT3, AOAH
intercellular and second messenger signaling	ERK5 signaling	1.67	YWHAH, MEF2D, RPS6KA5, RPS6KA1, MAP3K3, MAP3K2
nuclear receptor signaling	PPARα/RXRα activation	2.20	PLCD1, CAND1, SHC1, CYP2C9, MAP3K7, IRS1, TGFB2, IL1B, NR2C2, STAT5B, MAP2K1, PRKAG1
apoptosis	SAPK/JNK signaling	1.57	MAP4K3, SHC1, MAP3K7, IRS1, MAPK8IP3, MAP3K3, MAP3K2
glycan aynthesis and metabolism	N-glycan biosynthesis	1.51	EDEM2, WDFY3, DPM1, DPAGT1, UGT2B10

(A)



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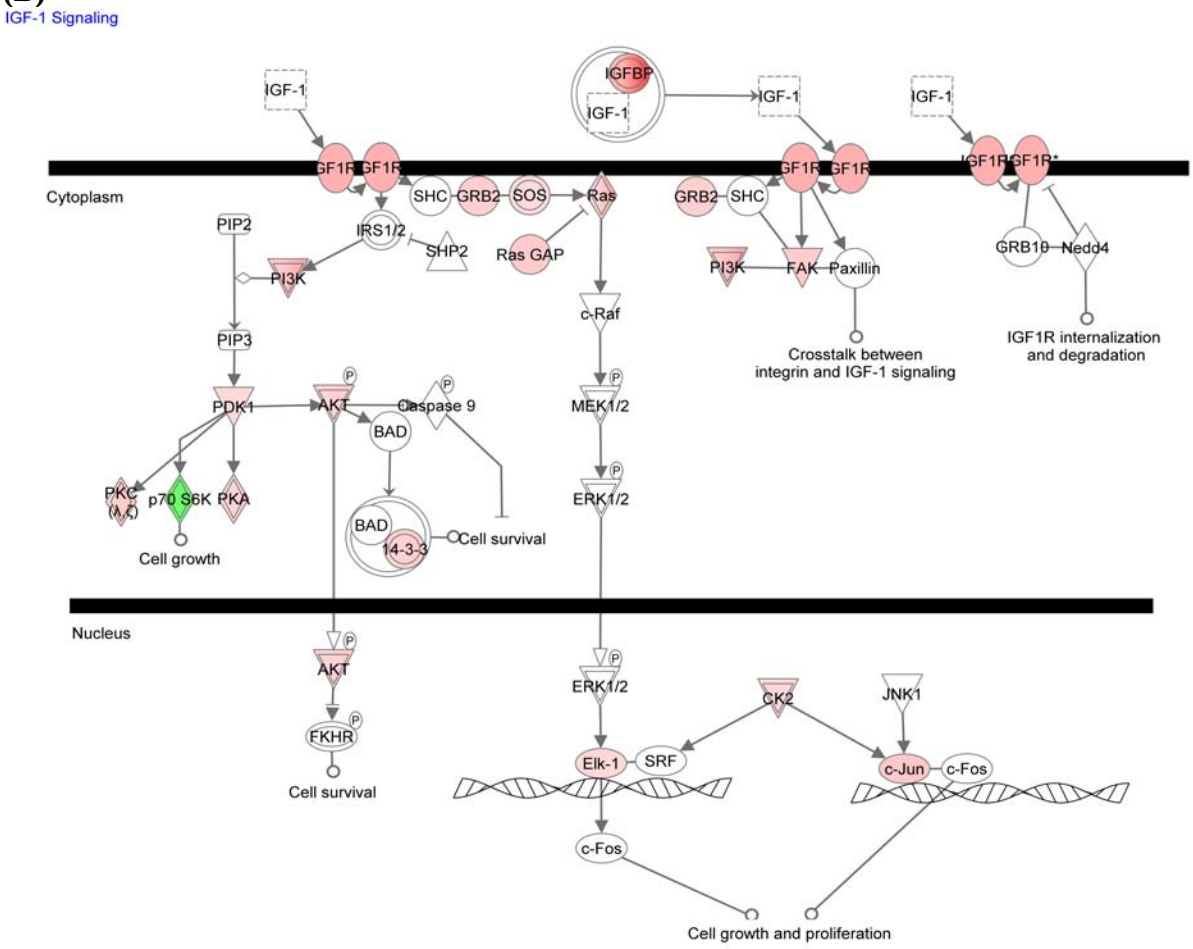


Figure 9: IPA canonical pathways that were significantly affected in DL at 91 dpc. (A) Diagram of IPA canonical pathway of IGF-1 signaling pathway with components encoded by genes up-regulated (down-regulated) in `MET´ highlighted in red (green) (B).

Table 18: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from DL at 91 dpc.

Categories	Ingenuity Canonical Pathways	-log (p-value)	Molecules
growth factor signaling	IGF-1 signaling	8.30	PRKACB, CTGF, PDPK1, KRAS, YWHAQ, PTK2, AKT1, JUN, PIK3CG, SOS1, IGF1R, CSNK2A1, MRAS, RPS6KB2, AKT3, PIK3R2, RASA1, IGFBP6, PIK3C2A, RRAS, GRB2, YWHAZ, RAC1, IGFBP5, PIK3R3, RRAS2, PRKCI, PRKAG2, PRKCH, ELK1, PRKAR1A
	HGF signaling	8.29	MAP3K11, CDC42, KRAS, MAP3K5, PTK2, ELF3, AKT1, JUN, MAP3K7, PIK3CG, SOS1, MRAS, AKT3, PIK3R2, PRKD1, PRKCA, MAP3K2, MAP2K7, PIK3C2A, RRAS, GRB2, RAC1, MAPK9, MAPK12, PIK3R3, RRAS2, PRKCI, GAB1, CDKN1A, PRKCH, ELK1, MAP3K3
cellular growth, Proliferation development	germ cell-sertoli cell junction signaling	9.92	MAP3K11, CDC42, AXIN1, DIRAS3, ACTA2, PVRL3, MLLT4, PDPK1, KRAS, MAP3K5 PTK2, TGFBR2, AKT1, RHOB, SORBS1, MAP3K7, PPAP2B, PIK3CG, MRAS, PIK3R2, RHOF, ACTC1, RAB8B, MAP3K2, ITGB1, PAK4, MAP2K7, PIK3C2A, RRAS, TJP1, RHOC, ITGA2, RAC1, MAPK9, MAPK12, ACTG1, PIK3R3, RRAS2, RHOQ, RND3, PAK3, A2M, MAP3K3, FNBP1, CTNND1
	PI3K/AKT signaling	8.09	GAB2, RELA, PPP2R2A, PDPK1, INPPL1, KRAS, MAP3K5, BCL2, PTEN, YWHAQ, IKBKG, AKT1, PIK3CG, SOS1, MRAS, RPS6KB2, AKT3, PIK3R2, TP53, ITGB1, RRAS, GRB2, PPP2R5D, ITGA2, RAC1, YWHAZ, PPP2R5A, PIK3R3, PPP2CB, RRAS2, GAB1, PPP2R4, CDKN1A, CDKN1B, PPP2R5E, PPP2R1B
	FAK signaling	7.82	FYN, ACTA2, PDPK1, KRAS, PTEN, PTK2, AKT1, PIK3CG, SOS1, MRAS, AKT3, PIK3R2, VCL, ACTC1, EGFR, ITGB1, PAK4, PIK3C2A, RRAS, GRB2, ITGA2, RAC1, ACTG1, PIK3R3, RRAS2, TLN2, PAK3, CAPN2, TNS1

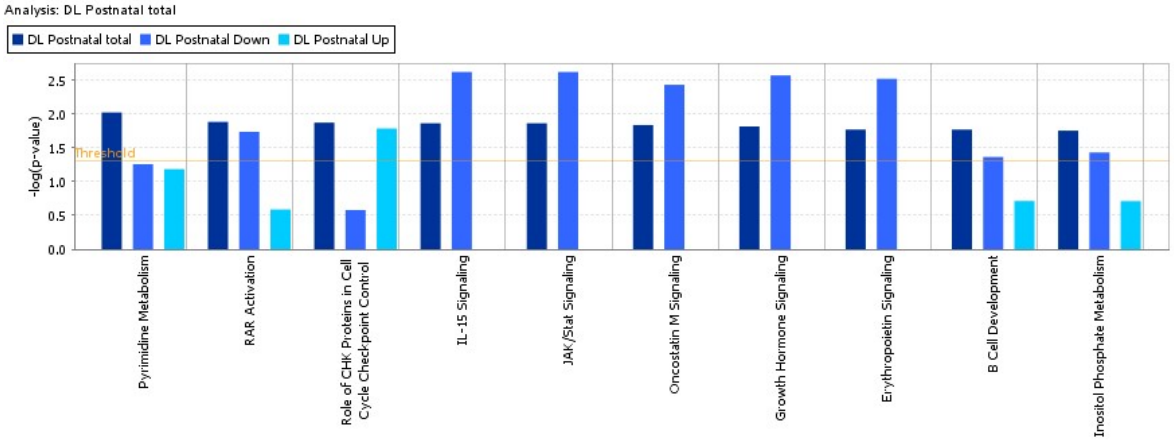
Table 18: (Continue) Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from DL at 91 dpc.

Categories	Ingenuity Canonical Pathways	-log (p-value)	Molecules
cellular growth, Proliferation development	integrin signaling	7.16	RAP2B, FYN, MAP3K11, CDC42, DIRAS3, ACTA2, ITGA8, PPP1CB, KRAS, PTEN, NCK2, PTK2, MYLK, AKT1, RHOB, ITGA9, PIK3CG, SOS1, ITGAV, MRAS, CAV1, AKT3, PIK3R2, VCL, RHOF, ACTC1, ITGB1, PAK4, PIK3C2A, GRB2, RRAS, RHOC, ITGA2, RAC1, ACTG1, PIK3R3, WIPF1, TLN2, RRAS2, RHOQ, RND3, PAK3, PPP1R12A, ITGA1, CAPN2, FNBP1, RAP2A
humoral immune respones	HMGB1 signaling	11.00	RELA, ICAM1, CDC42, DIRAS3, KRAS, AKT1, JUN, CCL2, HMGB1, RHOB, PIK3CG, MRAS, AKT3, PIK3R2, SERPINE1, TNFRSF1B, RHOF, MAP2K7, PIK3C2A, RHOC, RRAS, MYST4, RAC1, MAPK9, IL1R1, MAPK12, PIK3R3, KAT2B, TLR4, RRAS2, RHOQ, RND3, MYST3, ELK1, FNBP1
apoptosis	PTEN signaling	9.36	RELA, CDC42, PDPK1, INPPL1, KRAS, BCL2, PTEN, PTK2, IKBKG, AKT1, BMPR1A, PIK3CG, SOS1, MRAS, CSNK2A1, RPS6KB2, AKT3, PIK3R2, PDGFRB, EGFR, ITGB1, RRAS, GRB2, ITGA2, RAC1, PIK3R3, GHR, RRAS2, CBL, CDKN1A, CDKN1B, BCL2L11
intercellular and second messenger signaling	glucocorticoid receptor signaling	8.87	PRKACB, HSPA14, HSPA1A, PBX1, SMARCD2, KRAS, MNAT1, TGFBR2, IKBKG, HMGB1, PIK3CG, MRAS, SERPINE1, SMAD5, MED1, RRAS, RAC1, GTF2F1, MAPK12, PIK3R3, HSPA8, KAT2B, NCOA1, NR3C2, UBE2I, RELA, ICAM1, POLR2B, SLPI, NR3C1, SMARCA4, BCL2, JUN, AKT1, CCL2, PPP3CB, MAP3K7, SOS1, AKT3, NCOR1, SUMO1, PIK3R2, NOS2, STAT1, TAF2, PPP3CA, GTF2H3, MAP2K7, TAF6, PIK3C2A, TAF15, GRB2, IL10, MAPK9, CEBPB, NFATC4, RRAS2, CDKN1A, NRIP1, ELK1, A2M

At 91 dpc canonical pathways analysis revealed highly significant pathways (Figure 9A), namely HMGB1 signaling, germ cell-sertoli cell junction signaling, PTEN signaling, glucocorticoid receptor signaling, IGF-1 signaling, HGF signaling, ILK signaling, PI3K/AKT signaling, FAK signaling, integrin signaling. The categories of canonical pathway were consistently affected growth factor signaling, cellular growth, proliferation development, humoral immune respones, apoptosis, intercellular and second messenger signaling (Figure 9B). IGF-1 signaling (Figure 9B) was the major affected pathway with 31 regulated genes (PRKACB, CTGF, PDPK1, KRAS, YWHAQ, PTK2, AKT1, JUN, PIK3CG, SOS1, IGF1R, CSNK2A1, MRAS, RPS6KB2, AKT3, PIK3R2, RASA1, IGFBP6, PIK3C2A, RRAS, GRB2, YWHAZ, RAC1, IGFBP5, PIK3R3, RRAS2, PRKCI, PRKAG2, PRKCH, ELK1, PRKAR1A) showed in Table 18. We selected 4 up-regulation genes (AAK1, Smad5, MAP3K7 and IGFBP5) for validation.

At postnatal stage, the canonical pathways analysis showed highly significant pathways (Figure 10A), growth hormone signaling, erythropoietin signaling, oncostatin M signaling, B cell development, pyrimidine metabolism, RAR activation, role of CHK proteins in cell cycle checkpoint control, JAK/stat signaling, IL-15 signaling, inositol phosphate metabolism (Figure 10A). The categories of canonical pathway were consistently affected growth factor signaling, cellular growth, proliferation development, nucleotide metabolism, nuclear receptor signaling, cellular stress and injury, intercellular and second messenger signaling, apoptosis, metabolism of complex lipids (Table 19). We selected 3 genes for validation, including with IGF1, SOCS3 and MAPK3 and there were involved in IL-15 signaling, JAK/stat signaling, oncostatin M signaling, erythropoietin signaling, inositol phosphate metabolism and all 3 genes regulated in growth hormone signaling (Figure 10B). Pathways known to be affected on postnatal livers by RNA thoses are over expressed between `CON´ and `MET´ feed. Color of bars represented 3 data sets. Significance of enrichment threshold $p < 0.05$ is shown as yellow line. Bars that are above the line indicate significant enrichment of a pathway.

(A)



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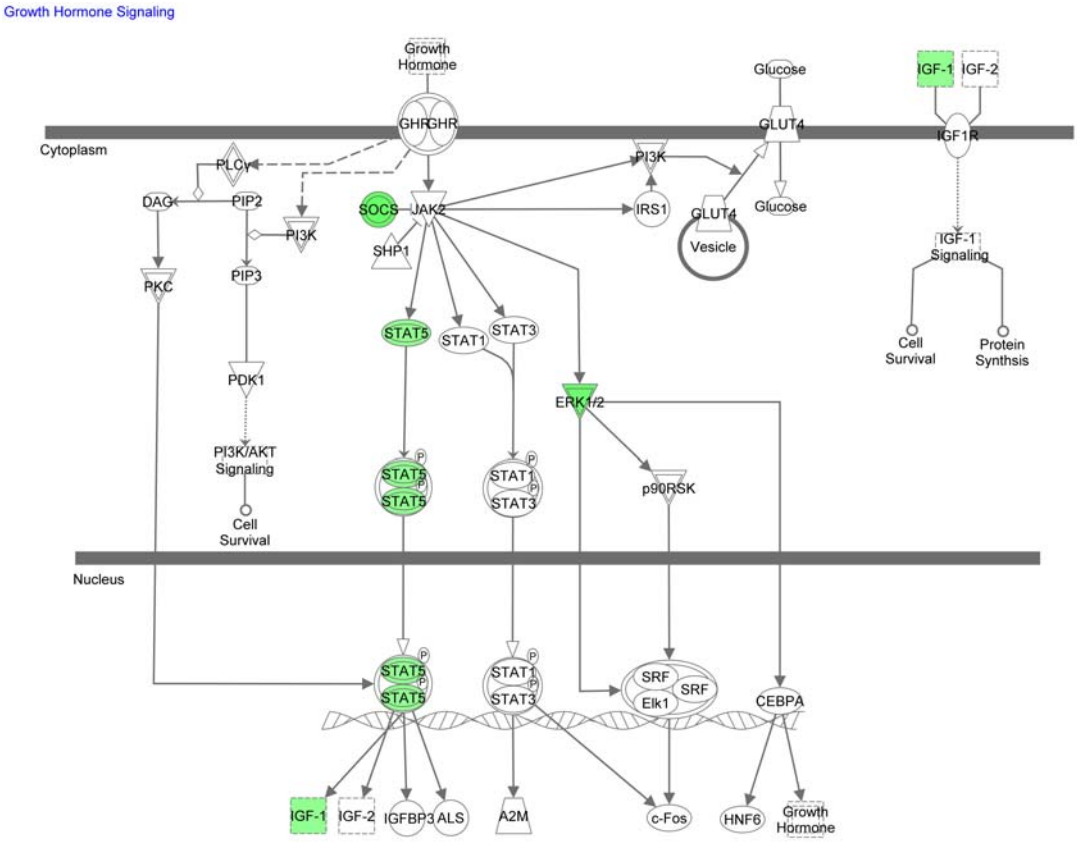


Figure 10: IPA canonical pathways that were significantly affected in DL at postnatal. (A) Diagram of IPA canonical pathway of growth hormone signaling pathway with components encoded by genes down-regulated in `MET´ highlighted in green (B).

Table 19: Summary of top 10 IPA Canonical pathways and lists of genes in those pathways obtained from DL at postnatal stage.

Categories	Ingenuity Canonical Pathways	-log(p-value)	Molecules
growth factor signaling	growth hormone signaling	1.81	SOCS3, STAT5A, IGF1, MAPK3
	erythropoietin signaling	1.77	SOCS3, STAT5A, CBL, MAPK3
cellular growth, proliferation development	oncostatin M signaling	1.83	STAT5A, MAPK3, TYK2
	B cell development	1.77	PTPRC, CD40, IGH@
nucleotide metabolism	pyrimidine metabolism	2.02	RRM2B, RRM2, MGC13098, CAD, CANT1, ENTPD7, RFC3
nuclear receptor signaling	RAR activation	1.88	STAT5A, CCNH, SMAD3, ERCC3, RDH12, MAPKAPK2, CRABP1
cellular Stress and Injury	role of CHK proteins in cell cycle checkpoint control	1.87	HUS1, CDK2, RFC3
intercellular and second messenger signaling	JAK/stat signaling	1.86	SOCS3, STAT5A, MAPK3, TYK2
apoptosis	IL-15 signaling	1.86	STAT5A, MAPK3, TYK2, BCL2
metabolism of complex lipids	inositol phosphate metabolism	1.75	CDK8, MAPK3, GRK6, IMPA2, PIP4K2B, CDK2

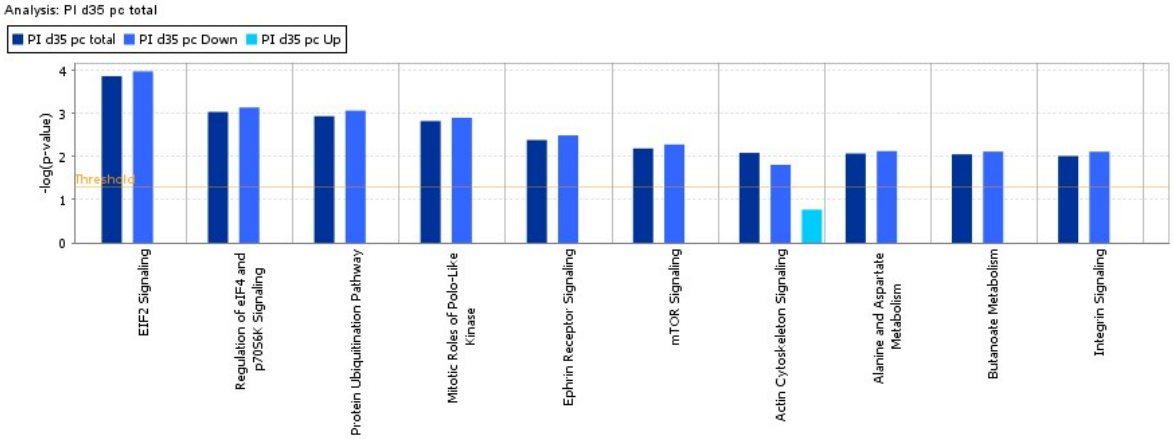
4.4.2 IPA pathway analysis of gene functions in PI

At 35 dpc canonical pathways analysis revealed highly significant pathways (Figure 11A), namely EIF2 signaling, regulation of eIF4 and p70S6K signaling, protein ubiquitination pathway, mitotic roles of polo-like kinase, ephrin receptor signaling, mTOR signaling, actin cytoskeleton signaling, alanine and aspartate metabolism, butanoate metabolism, integrin signaling. The categories of canonical pathway were consistently affected cellular growth, proliferation development, organismal growth and development, cell cycle regulation, amino acid metabolism, carbohydrate metabolism, intercellular and second messenger signaling (Table 20). The regulation of actin-based mobilitz by Rho at 35 dpc (Figure 11B) was the important canonical pathway influenced biological functions; there were 10 genes differentially expressed between `CON´ and `MET´ diet group. We selected 3 genes for validation, including with PFN, PAK and Rho there were involved in actin cytoskeleton signaling, ephrin receptor signaling, mTOR signaling (Table 20).

Table 20: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from PI at 35 dpc.

Categories	Ingenuity Canonical Pathways	-log(p-value)	Molecules
cellular growth, proliferation development	EIF2 signaling	3.86	EIF2S3, PPP1CC, EIF4G2, EIF1AX, PIK3R1, EIF5, KRAS, EIF4A2, EIF3E, EIF2A
	regulation of eIF4 and p70S6K signaling	3.03	EIF2S3, EIF4G2, EIF1AX, PIK3R1, ITGA2, KRAS, EIF4A2, EIF3E, PPP2R5E, EIF2A
	mTOR signaling	2.19	EIF4G2, RND3, RHOT1, DIRAS3, PIK3R1, KRAS, EIF4A2, HIF1A, EIF3E, PPP2R5E
organismal growth and development	ephrin receptor signaling	2.38	RAP1B, GNAI3, ACTR2, ACTR3, PAK2, PTPN11, PTPN13, CFL2, ITGA2, GNAI1, KRAS, EFNB3
	actin cytoskeleton signaling	2.08	TMSL3, PPP1CC, ACTR2, PAK2, PFN1, PIK3R1, ITGA2, KRAS, ACTR3, CFL2, MYH9, PPP1R12A, NCKAP1
	integrin signaling	2.01	RAP1B, PPP1CC, ACTR2, ACTR3, PAK2, RND3, RHOT1, DIRAS3, PIK3R1, ITGA2, PPP1R12A, KRAS
cell cycle regulation	mitotic roles of polo-like kinase	2.82	PLK4, CCNB2, HSP90AA1, PPP2R5E, CDK1, KIF11, STAG2
amino acid metabolism	alanine and aspartate metabolism	2.07	NARS, ABAT, ASS1, PDHX, DARS
carbohydrate metabolism	butanoate metabolism	2.05	ALDH1A1, ABAT, AUH, DBT, DCXR, L2HGDH
intercellular and second messenger signaling	protein ubiquitination pathway	2.94	USP15, UBR2, USP9X, UBE2V2, USP1, PSMD5, UBE3A, UBE4A, USP44, PSMC6, PSMD12, UCHL5, HSP90AA1, UBE2D3

(A)



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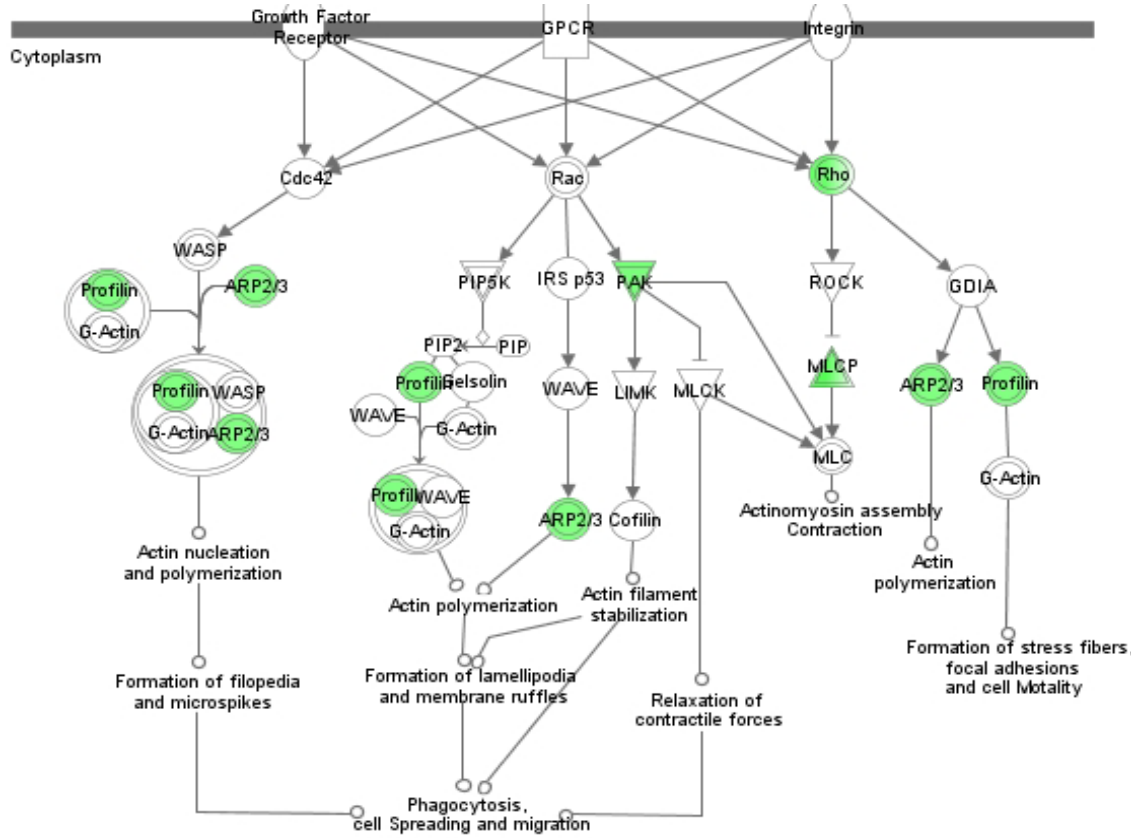
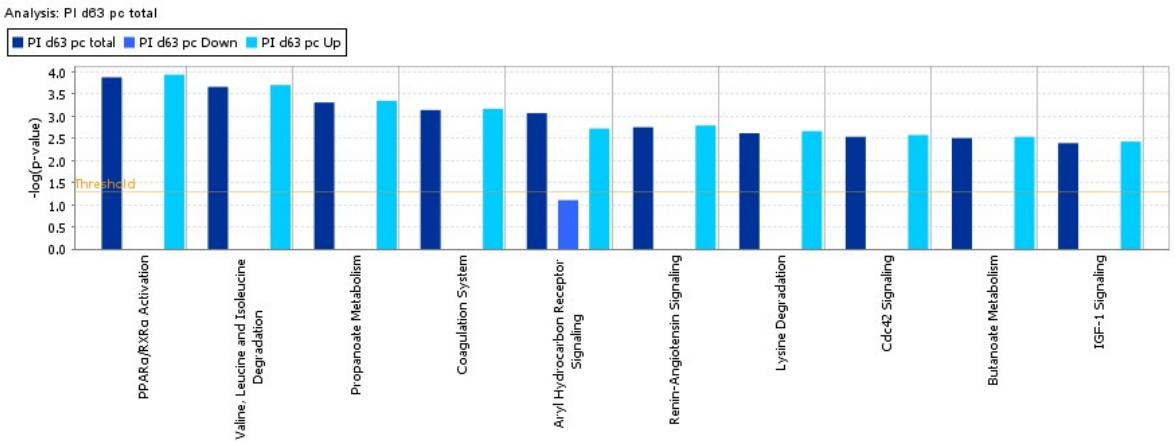


Figure 11: IPA canonical pathways that were significantly affected in PI at 35 dpc. (A) Diagram of IPA canonical pathway of actin-based mobilitz by Rho pathway with components encoded by genes down-regulated in `MET´ highlighted in green (B).

At 63 dpc canonical pathways analysis revealed highly significant pathways (Figure 12A), namely PPARα/RXRα activation, valine, leucine and isoleucine degradation, propanoate metabolism, coagulation system, aryl hydrocarbon receptor signaling, renin-angiotensin signaling, lysine degradation, Cdc42 signaling, butanoate metabolism, IGF-1 signaling. The categories of canonical pathway were consistently affected nuclear receptor Signaling, amino acid metabolism, carbohydrate metabolism, cellular stress and injury, apoptosis, growth factor signaling, amino acid metabolism, cellular growth, proliferation development, carbohydrate metabolism (Table 21). The IGF-1 signaling at 63 dpc (Figure 12B) was the important canonical pathway influenced biological functions; there were 14 genes (PRKACB, YWHAE, MAPK1, YWHAZ, PRKAR2A, IGFBP2, PRKAG1, FOS, JUN, PRKAR2B, PIK3C3, IGF1R, PRKAG2, RASA1) differentially expressed between `CON´ and `MET´ (Table 21).

(A)



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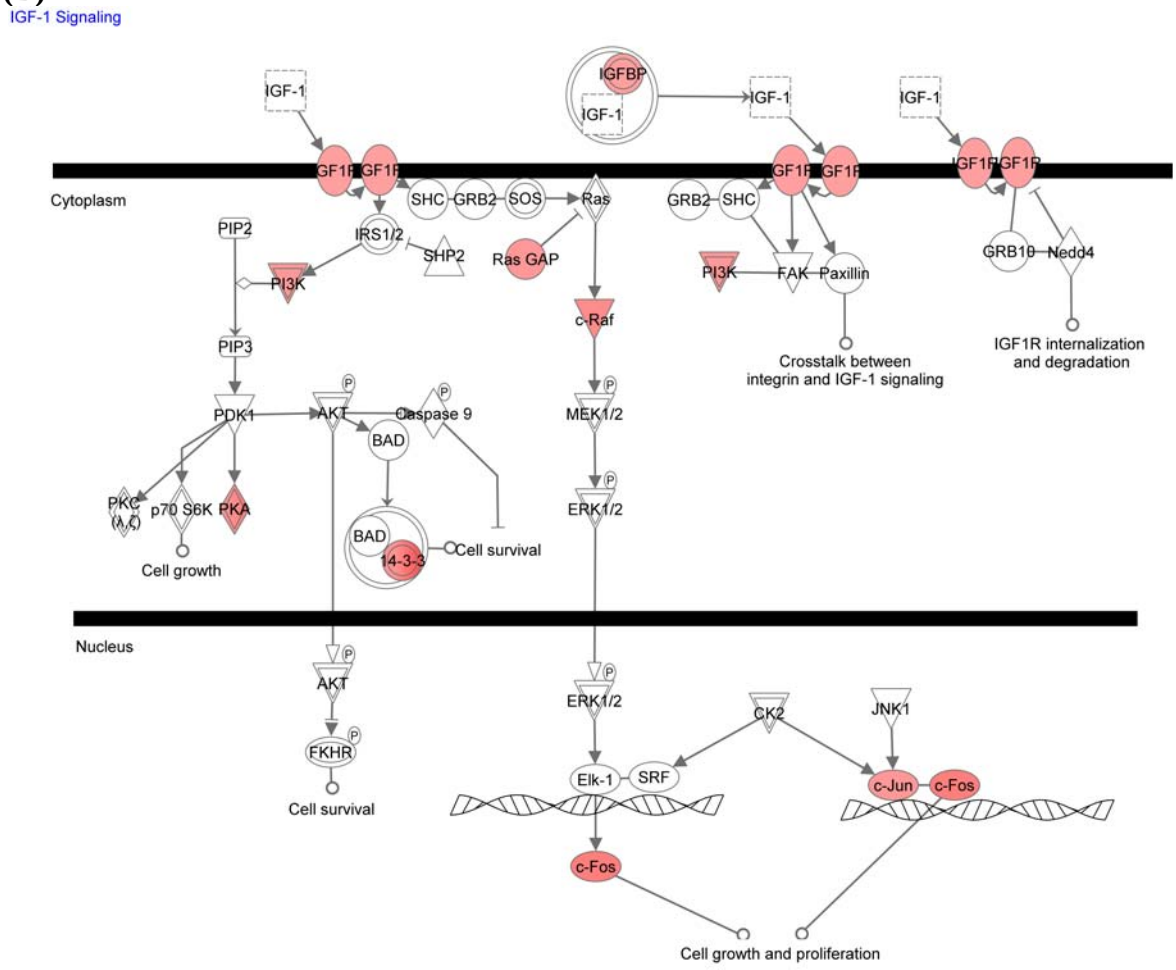


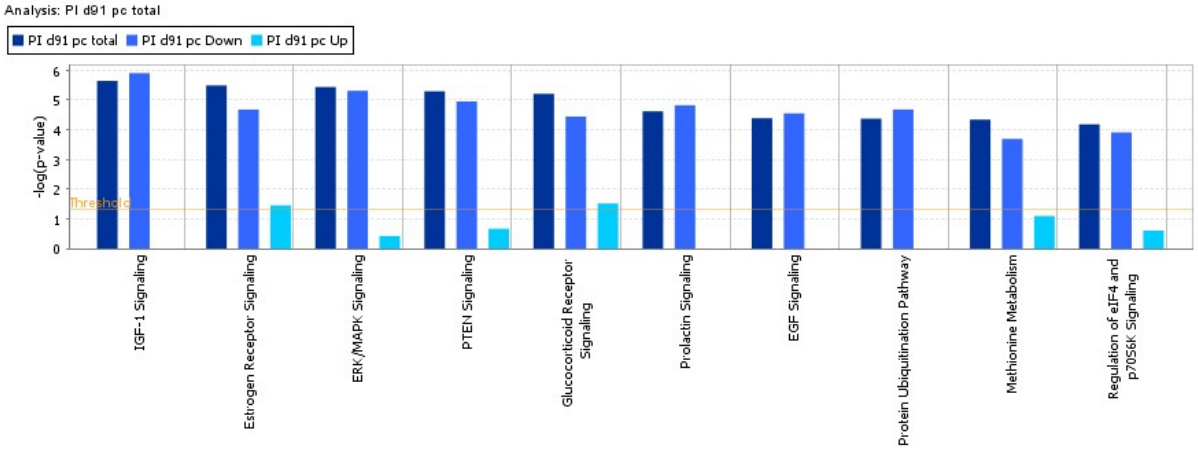
Figure 12: IPA canonical pathways that were significantly affected in PI at 63 dpc. (A) Diagram of IPA canonical pathway of IGF-1 signaling pathway with components encoded by genes up-regulated in `MET´ highlighted in red (B).

Table 21: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from PI at 63 dpc.

Categories	Ingenuity Canonical Pathways	-log (p-value)	Molecules
nuclear receptor signaling	PPARα/RXRα activation	3.87	PRKACB, MAPK1, CYP2C9, PRKAB2, PDIA3, MAP4K4, JAK2, ACVR2B, ABCA1, PRKAG1, JUN, MAP3K7, LPL, PRKAA1, ACAA1, MED1, CKAP5, PRKAR2A, GNAQ, CAND1, ACADL, PRKAR2B, PRKAG2, HSP90AA1, PPARGC1A
amino acid metabolism	valine, leucine and isoleucine degradation	3.66	ACAA1, ACADSB, BCAT1, HIBCH, ACADL, ALDH1A1, ACADVL, MCEE, ACAT1, DBT, MCCC1, HSD17B4, ACADM, ALDH7A1
carbohydrate Metabolism	propanoate metabolism	3.31	ACAA1, ACADSB, HIBCH, SUCLA2, ACADL, ALDH1A1, DHCR24, ACADVL, ACSS2, MCEE, ACAT1, ACADM, ALDH7A1
cellular stress and injury	coagulation system	3.13	KNG1 (includes EG:3827), SERPINC1, SERPINA5, F9, FGA, A2M, SERPINF2, FGG, SERPIND1
apoptosis	aryl hydrocarbon receptor signaling	3.06	GSTA2, MAPK1, MED1, CDK6, BAX, FOS, RB1, CCNA2, PTGES3 (includes EG:10728), ALDH1A1, JUN, NCOA2, MGST2, GSTA4, HSP90AA1, NRIP1, CDKN1B, NFE2L2, ATR (includes EG:545), ALDH7A1
growth factor signaling	IGF-1 signaling	2.39	PRKACB, YWHAE, MAPK1, YWHAZ, PRKAR2A, IGFBP2, PRKAG1, FOS, JUN, PRKAR2B, PIK3C3, IGF1R, PRKAG2, RASA1
	renin-angiotensin signaling	2.75	PRKACB, MAPK1, ITPR2, GNAQ, PRKAR2A, MAPK9, JAK2, PRKAG1, ATF2, FOS, JUN, PRKAR2B, PIK3C3, PRKAG2, STAT1, AGTR2
amino acid metabolism	lysine degradation	2.61	AASDHPPT, ACAA1, CASP3, FGL2, IDE, CLPP, PLOD1, WHSC1L1, PEPD, ALDH1A1, CTSS, SENP6, ACAT1, F9, DBT, YME1L1, LAP3, HSD17B4, USP25, ALDH7A1, PSEN1
cellular growth, proliferation development	Cdc42 signaling	2.53	EXOC1, MAPK9, EXOC6, MYL1, ATF2, FOS, IQGAP2, ACTR3, JUN, CFL2, ARPC4, PPP1R12A, EXOC5, ARPC1A, PARD3, RASA1
carbohydrate metabolism	butanoate metabolism	2.50	SDHA, BDH2, SDHB, BDH1, ALDH1A1, ACAA1, AACS, ACAT1, DBT, HSD17B4, ALDH7A1

At 91 dpc canonical pathways analysis revealed highly significant pathways (Figure 13A), namely IGF-1 signaling, estrogen receptor signaling, ERK/MAPK signaling, PTEN signaling, glucocorticoid receptor signaling, prolactin signaling, EGF signaling, protein ubiquitination pathway, methionine metabolism, regulation of eIF4 and p70S6K signaling. The categories of canonical pathway were consistently affected growth factor signaling, intercellular and second messenger signaling, nuclear receptor signaling, cellular growth, proliferation development, amino acid metabolism, apoptosis, cytokine signaling (Table 22). The methionine metabolism at 91 dpc (Figure 13B) was the important canonical pathway influenced biological functions; there were 10 genes differentially expressed between `CON´ and `MET´ (Table 22).

(A)



(B)

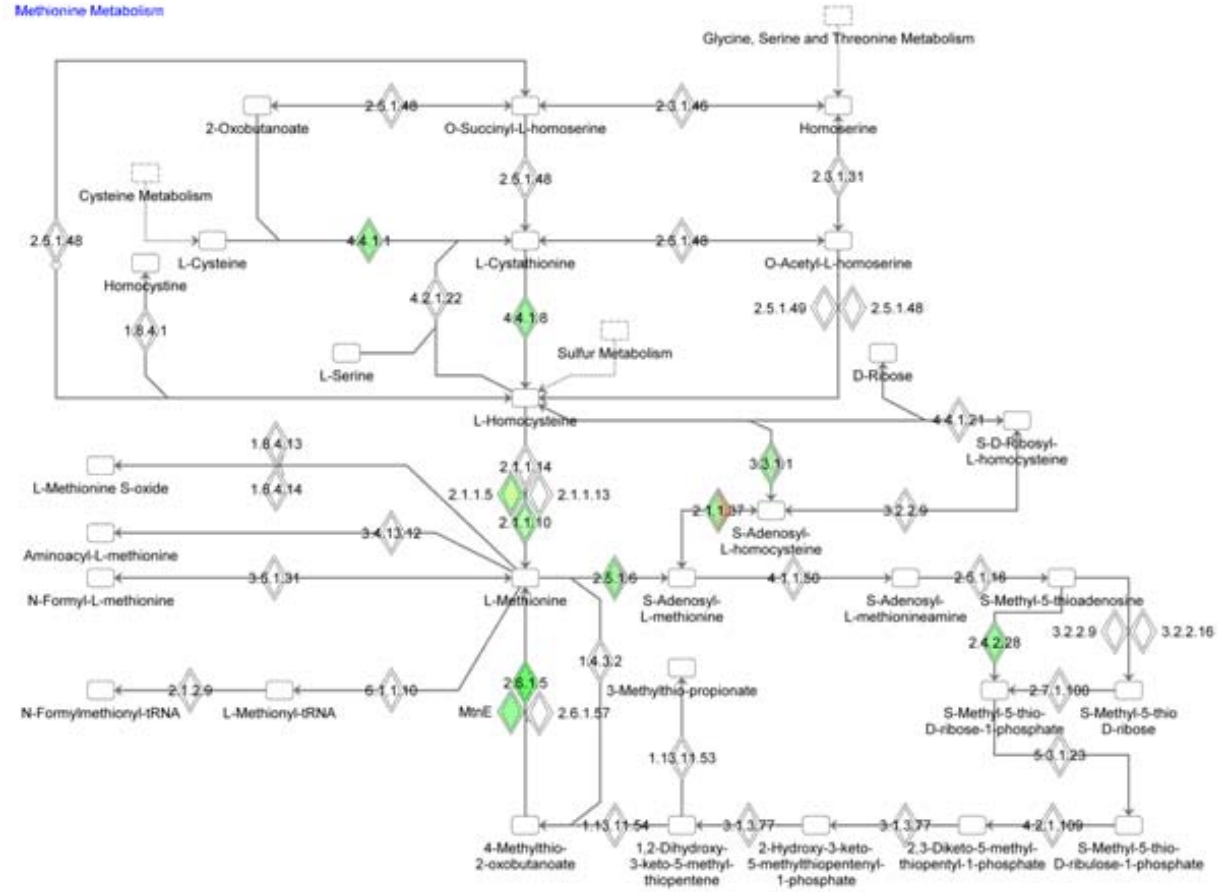


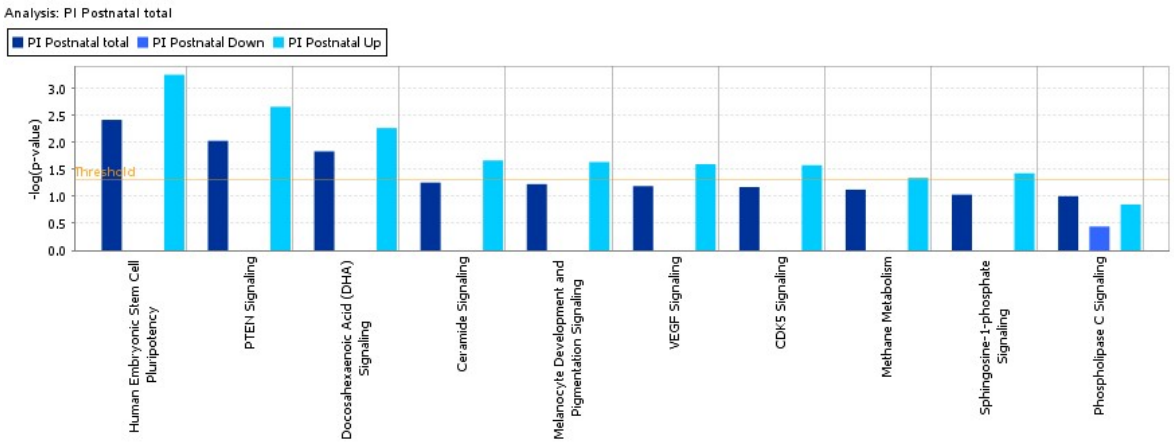
Figure 13: IPA canonical pathways that were significantly affected in PI at 91 dpc. (A) Diagram of IPA canonical pathway of methionine pathway with components encoded by genes down-regulated in `MET´ highlighted in green (B).

Table 22: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from PI at 91 dpc.

Categories	Ingenuity Canonical Pathways	-log (p-value)	Molecules
growth factor signaling	IGF-1 signaling	5.64	MAPK1, PRKAR2A, RAC1, HRAS, PDPK1, PRKAG1, IGFBP2, GRB10, SHC1, CSNK2A2, RRAS2, JUN, PRKAR2B, PTPN11, IRS1, CSNK2A1, IGF1R, PIK3R2, IGFBP1, CSNK2B, MAP2K1
	EGF signaling	4.38	SHC1, CSNK2A2, JUN, MAPK1, CSNK2A1, HRAS, CSNK2B, STAT3, PIK3R2, STAT1, MAP2K1, EGFR
intercellular and second messenger signaling	ERK/MAPK signaling	5.44	RAP1B, PPP1CC, FYN, MAPK1, PTK2B, ETS2, H3F3B, HRAS, RAPGEF4, PRKAG1, MYC, SHC1, MKNK1, ATF4, PIK3R2, STAT1, MAP2K1, MYCN, SRC, PAK2, PPP2R5D, ITGA2, RAC1, PRKAR2A, ITGA5, STAT3, PPP2R1A, RRAS2, PRKAR2B, DUSP9, PPP1R12A
	glucocorticoid receptor signaling	5.21	MAPK1, HSPA1A, HRAS, GTF2E2, TAF7, TAF13, HSPA5, NR3C1, FGG, TGFBR2, HSPA4, SHC1, NFKBIA, POLR2C, PTGES3 (includes EG:10728), JUN, NCOA2, PCK2, BAG1, NCOR1, PIK3R2, SERPINE1, STAT1, STAT5B, MAP2K1, HSPA9, RAC1, TAT, STAT3, PCK1, TAF1, RRAS2, IL1RN, ERCC3, CDKN1A, NCOA1, NRIP1, A2M, UBE2I
	protein ubiquitination pathway	4.37	USP21 (includes EG:27005), B2M, USP45, UBE2L3, UBE3B, UBE2D2, HSPA5, USP2, USP7, HLA-A, STUB1, BAG1, UBE2V1, USP10, UBE2D3 (includes EG:7323), UBE2E3, AMFR, HLA-C, UBE2Q1, PSMD6, UBE2S, PSMD5, PSMD3, USP1, UBE3A, PSMD11, UBA1, CDC34 (includes EG:997), UBE2E1, UBE2I
nuclear receptor signaling	estrogen receptor signaling	5.49	SRC, MAPK1, HRAS, H3F3B, RBM9, HNRNPD, PCK1, TAF7, TAF13, NR3C1, SHC1, HDAC3, TAF1, POLR2C, RRAS2, PCK2, NCOA2, ERCC3, NCOA1, NCOR1, IGFBP1, NRIP1, MAP2K1
cellular growth, proliferation development	regulation of eIF4 and p70S6K signaling	4.18	EIF2S3, EIF4EBP2, MAPK1, PPP2R5D, ITGA2, RAC1, EIF2C2, HRAS, PDPK1, ITGA5, EIF4G1, SHC1, PPP2R1A, RRAS2, EIF3B, EIF1AX, IRS1, MKNK1, PIK3R2, MAP2K1
amino acid metabolism	methionine metabolism	4.34	TRDMT1, AHCYL1, BHMT, ALAS1, TAT, MAT2B, MAT2A, CTH, DNMT1, AHCY
apoptosis	PTEN signaling	5.29	MAPK1, ITGA2, RAC1, HRAS, PDPK1, BMPR2, ITGA5, CSNK2A2, SHC1, RRAS2, MAGI1, BMPR1A, CDKN1A, PDGFRA, CSNK2A1, PIK3R2, CSNK2B, CDKN1B, MAP2K1, EGFR
cytokine signaling	prolactin signaling	4.62	FYN, MAPK1, HRAS, STAT3, NR3C1, MYC, SHC1, JUN, RRAS2, PTPN11, IRS1, PIK3R2, PDK1, STAT5B, STAT1, MAP2K1

At postnatal stage canonical pathways analysis revealed highly significant pathways (Figure 14A), namely human embryonic stem cell pluripotency, PTEN signaling, docosahexaenoic acid (DHA) signaling, ceramide signaling, melanocyte development and pigmentation signaling, VEGF signaling, CDK5 signaling, methane metabolism, sphingosine-1-phosphate signaling, phospholipase C signaling. The categories of canonical pathway were consistently affected cellular growth, proliferation development, growth factor signaling, apoptosis, neurotransmitters and other nervous system signaling, cell cycle regulation, energy metabolism, intercellular and second messenger signaling, metabolism of complex lipids (Table 23). The PTEN signaling at postnatal stage (Figure 14B) was the important canonical pathway influenced biological functions; there were 10 genes differentially expressed between `CON´ and `MET´ (Table 23).

(A)



(B)

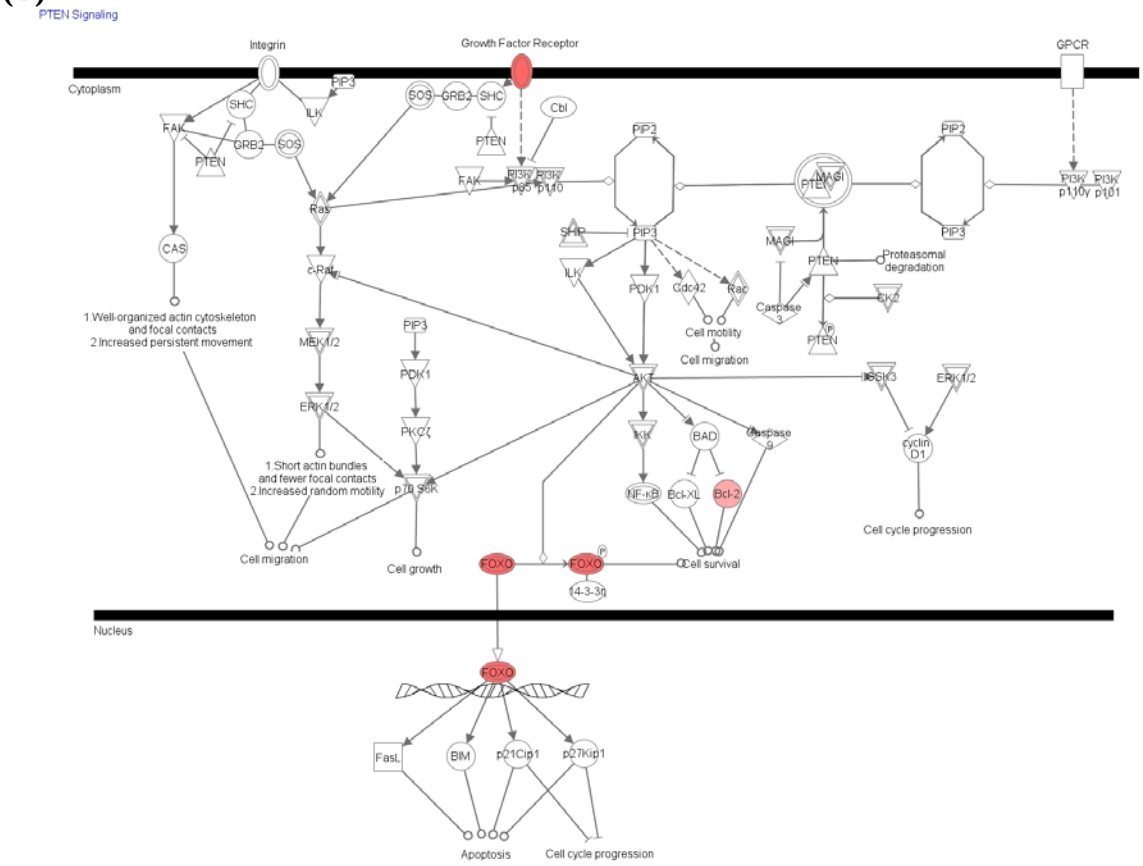


Figure 14: IPA canonical pathways that were significantly affected in PI at postnatal. (A) Diagram of IPA canonical pathway of PTEN signaling with components encoded by genes up-regulated in `MET´ highlighted in red (B).

Table 23: Summary of top 10 IPA canonical pathways and lists of genes in those pathways obtained from PI at postnatal stage.

Categories	Ingenuity Canonical Pathways	-log(p-value)	Molecules
cellular growth, proliferation development	human embryonic stem cell pluripotency	2.41	GNAS, PDGFRA, NGF, TCF3
	melanocyte development and pigmentation signaling	1.22	GNAS, BCL2
growth factor signaling	VEGF signaling	1.19	FOXO1, BCL2
apoptosis	PTEN signaling	2.03	FOXO1, PDGFRA, BCL2
	ceramide signaling	1.25	NSMAF, BCL2
neurotransmitters and other nervous system signaling	docosahexaenoic acid (DHA) signaling	1.83	FOXO1, BCL2
cell cycle regulation	CDK5 signaling	1.17	GNAS, NGF
energy Metabolism	methane metabolism	1.12	LPO
intercellular and Second Messenger Signaling	sphingosine-1-phosphate signaling	1.03	RHOQ, PDGFRA
metabolism of complex lipids	phospholipase C signaling	1.00	HDAC6, GNAS, RHOQ

4.5 Expression validation

To validate the gene expression results, 12 candidate genes from DL (Table 5) of the important canonical pathways at each stage were chosen for analysis by real-time PCR. Regulation of expression pattern from quantitative real-time PCR data showed down-regulation of BHMT, DNMT1, AMD1 at 35 dpc, IGFBP3, IGFBP5, SHC1 at 63 dpc, and SOCS3, IGF1, MAPK3 at postnatal stage; but AAK1, Smad5, MAP3K7, IGFBP5 showed up-regulation in `MET´compared to `CON´ at 91 dpc. For all genes exemplarily analysed quantitative real-time PCR confirmed the direction of differential regulation as obtained by microarray analysis (Table 24).

Table 24: Comparison of microarray data and quantitative real-time PCR results obtained of 12 selected transcripts in DL.

Stages	Gene name	Affymetrix microarray			Quantitative real-time PCR		
		p-value[1]	Regulation[2]	FC[3]	p-value[1]	Regulation[2]	FC[3]
35 dpc	BHMT	<0.001	down	2.3	0.020	down	1.6
	DNMT1	0.014	down	1.5	0.020	down	2.4
	AMD1	0.045	down	1.9	0.005	down	3.4
63 dpc	IGFBP3	0.020	down	1.8	0.012	down	1.5
	IGFBP5	0.040	down	2.4	0.037	down	1.4
	SHC1	0.050	down	1.7	0.061	down	1.3
91 dpc	AAK1	0.001	up	2.7	0.111	up	3.1
	Smad5	0.010	up	2.2	0.106	up	2.1
	MAP3K7	0.020	up	2.3	0.017	up	1.7
	IGFBP5	0.003	up	7.8	0.002	up	2.2
Postnatal	IGF1	0.025	down	2.0	0.019	down	2.0
	SOCS3	0.020	down	2.9	0.253	down	1.7
	MAPK3	0.030	down	2.7	0.206	down	1.2

[1]p-values obtained from microarray experiments and from real-time PCR after factorial normalization on RPL32

[2]direction of regulation

[3]FC=fold changes

*IGFBP5 found both in 35 and 91 dpc

To validate the gene expression results from PI, 12 candidate genes (Table 6) were chosen to be confirmed by quantitative real-time PCR. Regulation of expression pattern from quantitative real-time PCR data were shown down-regulation in PFN, Rho, PAK at 35 dpc and up regulation in IGFBP3, IGFBP5, IGF2R at 63 dpc. At 91 dpc were up-regulated in DNMT1 and down regulated in BHMT, MTAP. Three genes from postnatal stage were shown down-regulation in MAFG and up regulation in FOXO1 and BCL2. We used RPL32 to normalize expression value of all genes. Results of 12 genes in Table 8 were shown significantly and closely correlated to the corresponding expression profile data by both from microarray data and quantitative real-time PCR. The P values of the differences of transcript abundance between the `CON´ and `MET´ groups ranged <0.001 to 0.488 (Table 25).

Table 25: Comparison of microarray data and quantitative real-time PCR results obtained of 12 selected transcripts in PI.

Stages	Gene name	Affymetrix microarray			Quantitative rea-time PCR		
		p-value[1]	Regulation[2]	FC[3]	p-value[1]	Regulation[2]	FC[3]
35 dpc	PFN	0.027	down	1.7	<.0001	down	1.6
	PAK	0.037	down	1.8	0.488	down	1.1
	Rho	0.005	down	1.8	0.017	down	3.6
63 dpc	IGFBP3	0.048	up	1.6	0.004	up	1.4
	IGFBP5	0.042	up	1.4	0.045	up	1.9
	IGF2R	0.023	up	1.5	0.003	up	1.3
91 dpc	DNMT1	0.011	up	1.8	0.040	up	1.5
	BHMT	0.018	down	1.5	0.008	down	1.4
	MTAP	0.049	down	1.8	0.004	down	1.3
Postnatal	FOXO1	0.0117	up	2.6	0.024	up	1.5
	BCL2	0.0015	up	1.5	0.035	up	1.3
	MAFG	0.0036	down	2.4	0.083	down	1.3

[1]p-values obtained from microarray experiments and from real-time PCR after factorial normalization on RPL32

[2]direction of regulation

[3]FC=fold changes

4.6 Hepatic SAM and SAH concentration of sows and offspring

The results from HPLC analysis shown compares of SAM and SAH between `CON´ and `MET´ in sow and fetus from DL and PI were showed in Figure 15 and 16, respectively. SAM and SAH ratio between `CON´ and `MET´ from DL in Figure 15 demonstrated that similarly tendencies of SAM, SAH and SAM/SAH ratio on either 35 and 63 dpc. SAM, SAH and SAM/SAH ratio on 91 dpc were change and shown divergent tendencies. SAM/SAH ratio compares between `CON´ and `MET´ in sows and pooled of fetus liver were shown in Figure 15C and 15F, respectively. The SAM/SAH ratio in sows were higher in `CON´ than `MET´ at 35 and 91 dpc only SAM/SAH ratio at 63 dpc was lower in `CON´ than `MET´. In fetus, SAM/SAH ratio was lower in `CON´ than `MET´ on 63 and 91dpc.

SAM and SAH concentration between `CON´ and `MET´ in sow and fetus from DL in Figure 16 demonstrated that ratio of SAM/SAH in sows were lower in `MET´ than `CON´ that similarly tendencies on every stages (35 dpc, 7.95±0.48>4.14±0.67; 63 dpc, 5.54±1.39>3.76±0.41; 91 dpc, 3.84±1.60>3.65±1.19) were shown in Figure 16C. We compared SAM/SAH ratio between `CON´ and `MET´ in pooled of fetus liver were shown in Figure 16F. SAM/SAH ratio were lower in `CON´ more than `MET´ on every stages (35 dpc, 12.66<23.74; 63 dpc, 11.18<11.74; 91 dpc, 10.27<11.16).

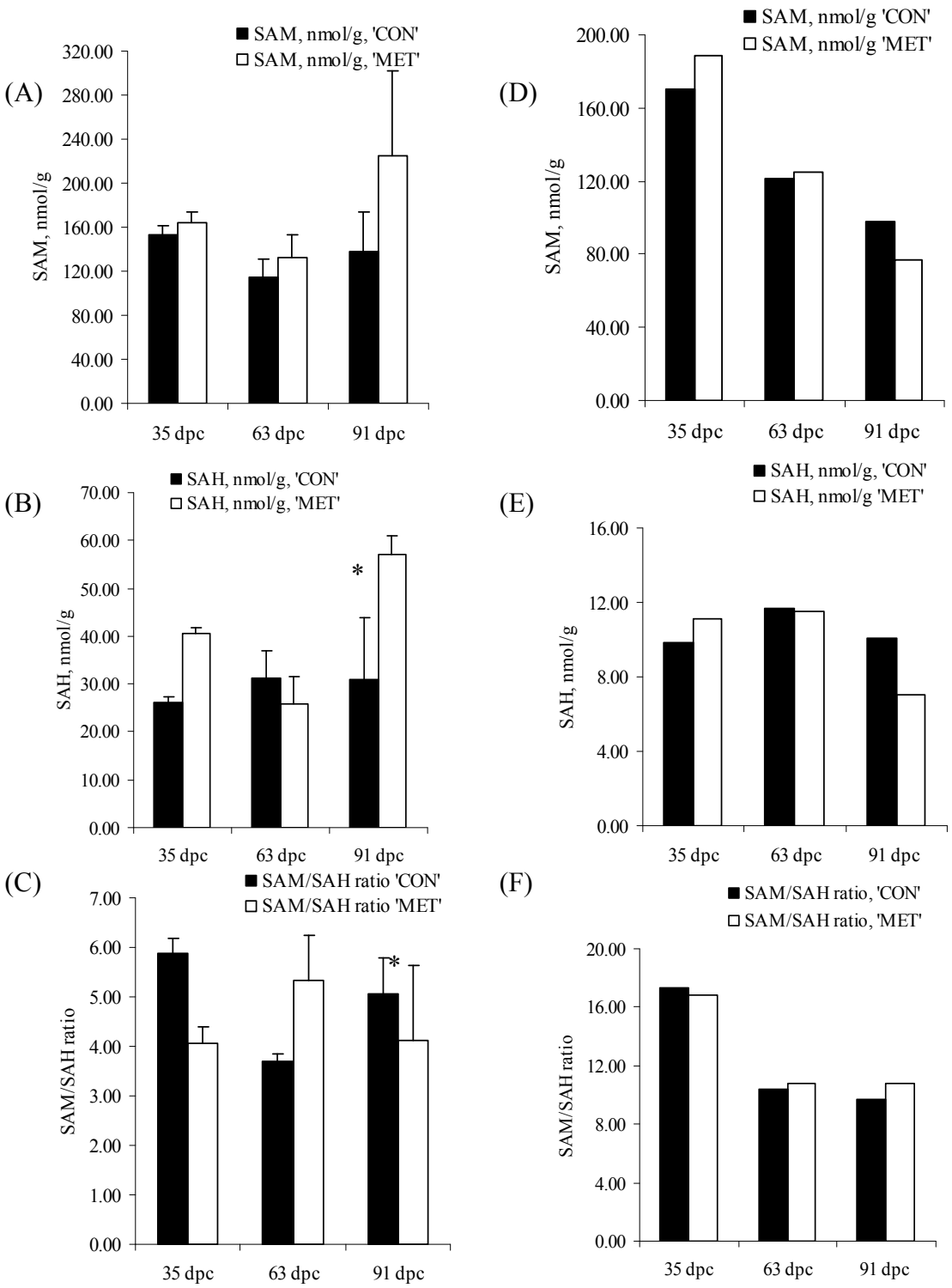


Figure 15: The concentration of SAM (A), SAH (B), and the SAM/SAH ratio (C) in DL sows and in pooled liver samples of DL fetuses (D, E, F), obtained at 35, 63, and 91 dpc in `MET´ and `CON´. Values obtained from sows are lsmeans± SE. * significant differences $p \leq 0.05$

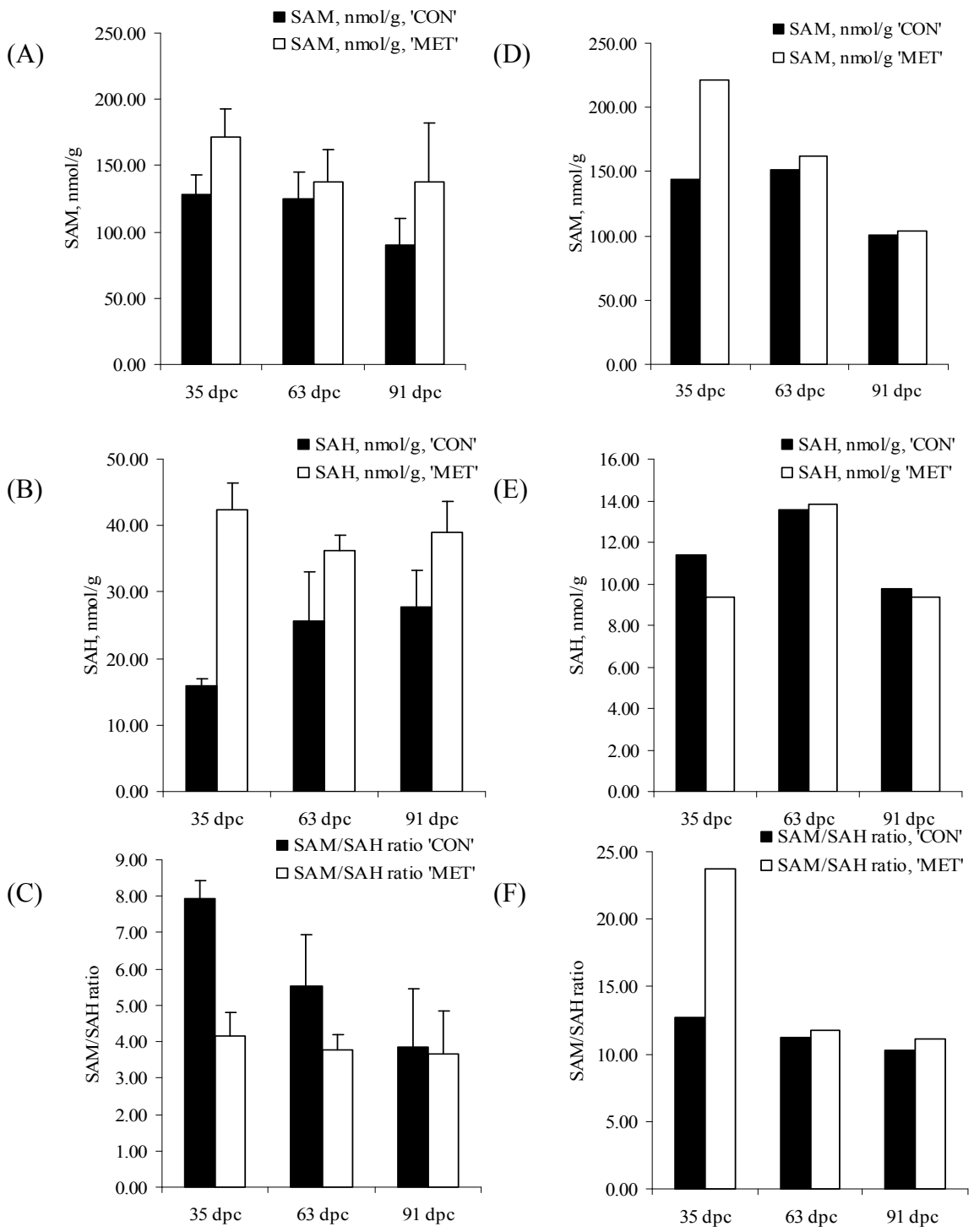


Figure 16: The concentration of SAM (A), SAH (B), and the SAM/SAH ratio (C) in PI sows and in pooled liver samples of PI fetuses (D, E, F), obtained at 35, 63, and 91 dpc in `MET´ and `CON´. Values obtained from sows are lsmeans± SE. * significant differences $p \leq 0.05$

4.7 Global DNA methylation analyses

The status of global DNA methylation was evaluated by using the Methylamp^{TM} Global DNA Methylation Quantification Ultra Kit for 5-methylcytosine (5-mc) detection in selected DNA samples from 3 stages of fetuses (35 dpc (n=74), 63 dpc (n=77), 91 dpc (n=79)). Levels of DNA methylation differed significantly due to treatment ($p < 0.05$; `CON´ > `MET´), breed ($p < 0.0001$; PI > DL) and prenatal stage ($p < 0.0001$; 91 dpc > 35 dpc > 63 dpc).

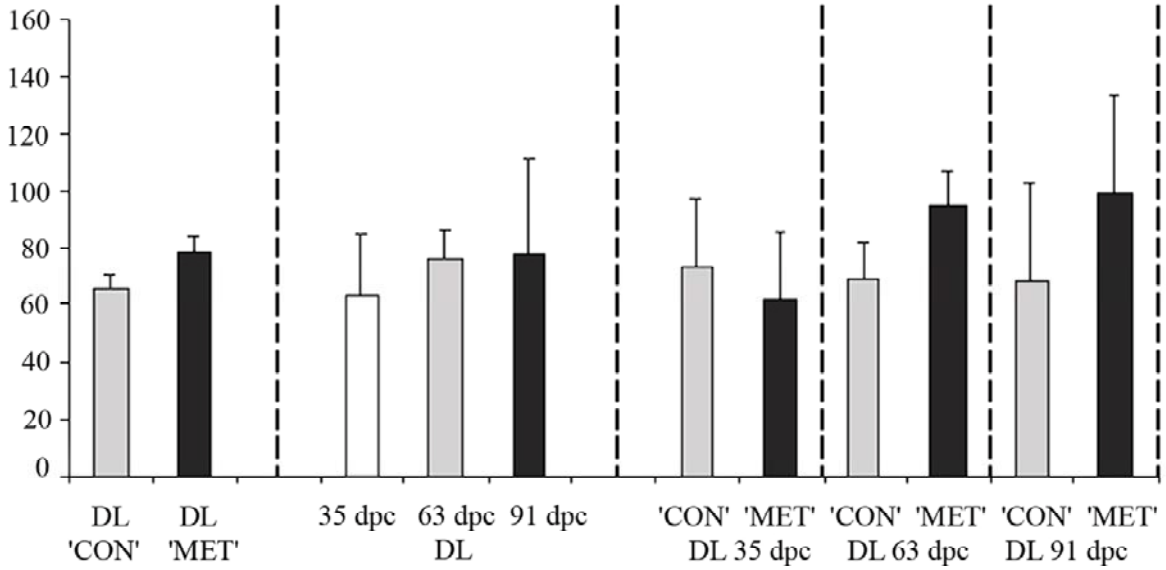


Figure 17: Methylation levels of genomic DNA between `CON´ and `MET´ in all stages and in different stage 35, 63 and 91 dpc from DL.

In DL the 5-mc mean scores of DNA in `CON´ (65.31±5.65) was significantly lower than those of DNA in `MET´ (78.80±5.75) averaged of all stages (Figure 17). Correspondingly the global DNA methyl incorporation was significantly higher in DNA from `MET´ than `CON´ at 63 dpc ('CON'; 69.06±12.53, `MET´; 94.71±12.59) and 91 dpc ('CON'; 68.43±34.03, 'MET'; 98.40±35.12) (Figure 17). However, at 35 dpc there was no significant difference in global DNA methylation in DL ('CON'; 73.92±22.87, 'MET'; 61.99±23.54) (Figure 17). Moreover, between different stages no significant differences were obtained in DL; numerically stage 35 dpc showed lowest methylation (62.98±22.13) followed by 63 dpc (75.89±10.76) and 91 dpc (77.30±33.85) (Figure 17). In PI, `CON´

showed higher levels of DNA methylation than `MET´; this was consistent at all prenatal stages (Figure 18). Lowest levels of DNA methylation were found at 63 dpc (Figure 18).

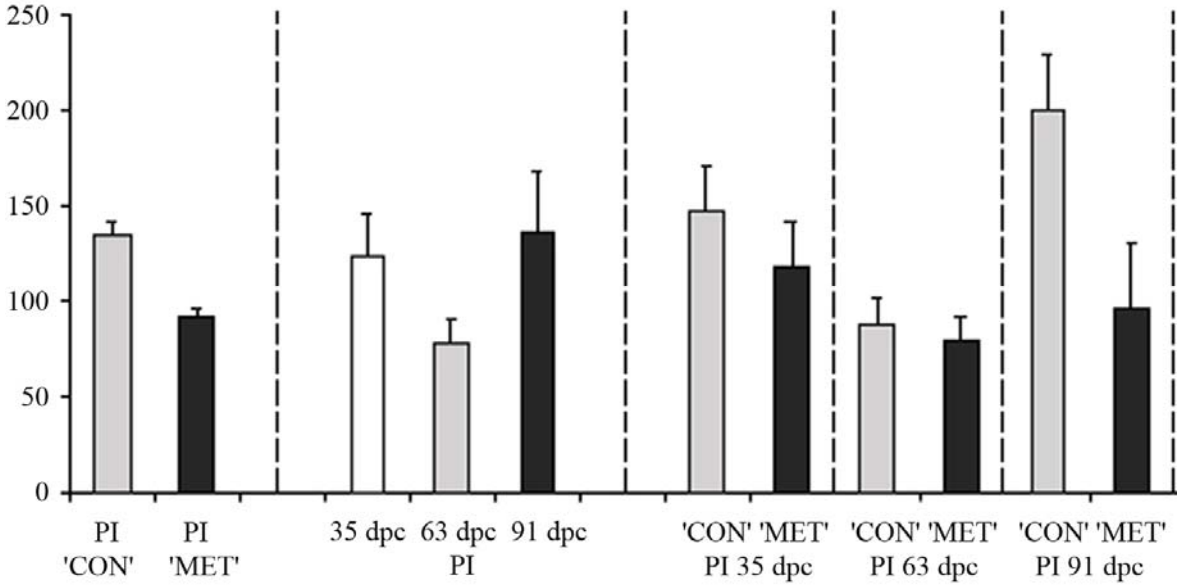


Figure 18: Methylation levels of genomic DNA between `CON´ and `MET´ in all stages and in different stage 35, 63 and 91 dpc from PI.

5. Discussion

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 at adulthood, including the metabolic syndrome. Pigs share similarity in metabolism, physiology, anatomy and genome with humans and are therefore a good approximation of the human condition. We evaluated hepatic genes expression changed due to impact of maternal methyl supplementation during the different stages of fetal development in pigs. We focused on the liver because it plays a central role in the regulation of the metabolic status (Ponsuksili et al., 2007). Expression patterns of liver tissue derived from German Landrace (DL) and Pietrain (PI) at 35, 63, 91 dpc and postnatal stage were examined. According to the hypothesis that the offspring's transcriptome is permanently regulated, we identified molecular pathways and candidate genes with relevance regarding the fetal initiation of postnatal growth.

5.1 Effects of the gestation diets on the offspring's phenotype

Maternal nutrition plays a critical role in fetal growth and development. Although considerable effort has been directed towards defining nutrient requirements of animals over the past 30 years, suboptimal nutrition during gestation remains a significant problem for many animal species (e.g., cattle, pigs, and sheep) worldwide. (1) Despite advanced prenatal care for mothers and fetuses, 5% of human infants born in the U.S. suffer from intrauterine growth retardation (IUGR). (2) Over the past decade, compelling epidemiological studies have linked IUGR with the etiology of many chronic diseases in adult humans and animals. (3) These intriguing findings have prompted extensive animal studies to identify the biochemical basis for nutritional programming of fetal development and its long-term health consequences.

The differing weights of the fetuses demonstrated that maternal `MET´ treatment affects the phenotype of the DL offspring at 35 ($P<0.001$), and slightly at 63 and 91 dpc in terms of body weight that was decreased in `MET´ (Table 2.). Independent of the diet weight of the fetuses was enormously increased between 63 dpc and 91 dpc. That corresponds with

results from another study on fetal growth in pigs where between d90 and d110 of gestation the weight of the fetuses was shown to increase cubically during this period (McPherson et al., 2004). Regarding the effect of `MET´ our finding was in accordance with Knipfel et al. (1975) who studied the fetal growth in sheeps. Fetal growth and amino acid patterns and postnatal behavior were alterated following methionine loading of ewes. This adverse effect of methionine might be due to the limited capacity or nonexistent mechanisms of the fetus for catabolizing sulfur amino acids. Various essential products of the transulfuration pathway for methionine catabolism reach the fetus following maternal catabolism. Under conditions where large doses of methionine or cysteine are given, for example, as stimulants to wool production, accumulation of methionine or its metabolites in the fetus might affect adversely fetal growth and development (Dowries et al., 1970). In mammalian, Zeisel (2006) indicated that an intake of methyl supplementation since fertilization can have a profound impact upon gene expression and these effect were being set in the post-implantation embryo (Reik and Walter, 2001; Robertson and Wolffe, 2000) and the early fetus in humans (Razin and Shemer, 1995), rats (Tsujiuchi et al., 1999), mice and cows (Dean et al., 2001; Wolff et al., 1998; Cooney et al., 2002).

The weight of our fetuses in PI offspring were statistically significant affected; however, in PI `MET´ and `CON´ had opposite effects compared to DL offspring. The results demonstrated that the genetic differences between more obese (DL) and more lean (PI) pigs are associated with opposed response to the experimental gestation diets with higher carcasses weight in `CON´ vs. `MET´ in obese pigs on the one hand and lower of carcasses weight in `CON´ < `MET´ in lean pigs on the other hand. This difference might be at the expense of the sows' weight and backfat thickness and might reflect differences in the requirements regarding the components of the one-carbon cycle of the sows and the fetuses of both breeds.

In the present study we have slaughtered pigs breed DL and PI at a mean age of 158 days at the abattoir of the FBN-Dummerstorf and carcass and meat quality data were collected according to guidelines of the Zentralverband der Deutschen Schweineproduktion e.V. (ZDS, 2004). In DL and PI postnatal growth performance of was higher in `CON´ > `MET´,

however only in PI carcasses weights were significant higher. Influences of maternal diet during early to mid-gestation on postnatal growth rate have also been observed by Bee (2004). The birth weight of fetuses from sows that received a standard diet grew slower ($P < 0.05$) during lactation and the growing-finishing period. Moreover, they had a lower ($P < 0.05$) gain to feed ratios, and had higher ($P < 0.05$) percentages of adipose tissue than pigs born from sows that take low-energy diet (Bee et al., 2004). Other studies of growth performance and carcasses quality in the pigs have demonstrated that maternal nutrition restriction during gestation has an effect on piglet birth weight, and that low birth weight is primarily associated with a reduced number of secondary muscle fibers (Dwyer et al., 1994). This finding was in agreement with previous reports from Bee (2004) by reported those maternal feeding regimen affected muscle fiber type distribution, whereas birth weight and gender affected muscle fiber area and Gentry et al. (2004) explained muscle fiber type percentages are influenced by environmental factors, genetics, nutrition, and exercise.

In contrast, Nissen et al (2003) reported sows were treated by standard diet gestation. One group of sows were fed ad libitum either from d 25 to 50 or from d 25 to 70 of gestation and another one were fed with (15 MJ of NE (2 kg)/d from d 1 to 90, then 24 MJ of NE (3.2 kg)/d from d 91 to 112, and again 15 MJ of NE/d from d 113 to 115 of gestation). Sows on those two experimental have no modulating effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring.

5.2 Expression Analysis

In view of the fact that `MET´ treatment contained supplementation of choline, betaine, folate, vitamin B12, vitamin B6 and methionine, which are interrelated at the point where homocystein is converted into methionine, changes in the expression of genes could be associated with methylation (Jeltsch, 2002; Niculescu and Zeisel, 2002; Zeisel, 2006; Selhub et al., 1991). Higher levels of dietary methionine might increase plasma homocysteine concentration, since methionine is the sole precursor of homocysteine.

Expression profiles were separated into three district clusters and the data show that clustering was according to stage, mother and diet; i.e. mother actually represents a stronger effect on the expression patterns than the diet. Accordingly, we decided to use $Y = \mu + diet + mother\ (diet)$ as the model for statistic analysis and to the list genes with differential expression. Maternal effects on the offspring hepatic expression patterns are due to the genetic relationship between mother and fetuses but also due to the maternal intra uterine environment. The expression profiles provided large list of affected genes at prenatal stages, where the direct effects of maternal supply to the fetus is most important. Whereas at postnatal stages, these direct effects are lacking and only effects are visible that represent a kind of metabolic memory, this phenomenon is termed nutritional (or fetal) programming. According to the hierarchical clustering of the expression profiles (Figure 4) indicating effects of the ontogenetic stage and maternal effects on the expression pattern being larger than the influence of nutrition at prenatal stage, one could suggest that direct short term effects on the expression pattern may be due to this environmental factor rather than the role of nutrition on the epigenetic regulation, that might result in the postnatal modifications of the expression patterns.

5.2.1 IPA pathway analysis to identification of gene functions in DL

In DL offspring we found a number of genes differentially expressed at 35 dpc with a fold change higher more than 1.5 with 13.9% of false discovery rate, most genes in list were down-regulated in the `MET´. At 63 dpc a lower number of genes were regulated, whereas at 91 dpc the highest number of regulated genes was found. At postnatal stage 367 genes were found differentially expressed depending on the gestation diets. Biological pathway identified by Ingenuity Pathway Analysis cover various metabolic and signaling pathways including methionine metabolism pathway at 35 dpc and IGF-1 signaling pathway both at 63 and 91 dpc. The canonical pathways that were consistently affected in all stages were involved in cellular growth, proliferation development and growth factor signaling. The pathways that are keys found from our work were the methionine metabolism, IGF-1 signaling pathway and growth hormone signaling that showed nutrition-mediated gene expression and are know to exhibit a strong association with growth development. These findings suggest that `MET´ diet acts as regulator of gene expression of metabolic genes in

early fetus development that might affect growth development in 63, 91 dpc and postnatal stages.

For validation of microarray chip data, we performed real-time PCR with the same RNA sample that were used for microarray experiments. In total, 12 target genes were tested by real-time PCR. RPL32 was used as a housekeeping control and the average fold change between microarray and real-time PCR was correlated.

Methionine metabolism pathway was found at 35 dpc as the highest significantly regulated canonical pathway. We found the expression of 10 genes which play roles in the methionine metabolism (TRDMT1, SRM, AMD1, AHCYL1, BHMT, ALAS1, TAT, MTR, MAT2B, DNMT1) to be significantly down-regulated. This pathway is the major source of methyl groups that influence DNA methylation and gene expression. Increasing levels of dietary methionine affects the metabolites and enzymes of the methionine metabolism which has been shown in rat liver (Finkelstein, 2000, Finkelstein 2007; Finkelstein and Martin, 1986) and in sheep liver (Xue and Snoswell, 1985). BHMT, DNMT1 and AMD1 were selected for confirmation of differential expression by real-time PCR. DNMT1 is a DNA methyltransferase and plays an important role in the regulation of gene expression. It is the predominant maintenance methyltransferase gene that is required to maintain DNA methylation patterns in mammalian cells (Fan et al., 2007; Park et al., 2004; Bird, 2002) during carcinogenesis, spermatogenesis (Omisanjo et al., 2007), gametogenesis and early embryonic development (Hajkova, 2002). These molecules keys were shown to be significantly lower expressed in `MET´ diet group compared to `CON´. It might be regulated by a methylation-sensitive DNA element. Methylation of DNA is an attractive system for feedback regulation of DNA methyltransferase as the final product of the reaction, methylated DNA, can regulate gene expression in cis (Andrew et. al., 1999). Betaine-homocysteine methyltransferase (BHMT) regulates homocysteine levels in the liver (Ji et al., 2007) and the reaction is the metabolic source of dimethylglycine (Finkelstein and Martin, 1986) and is linked to the homocysteine metabolism. BHMT transfers a methyl group from betaine to homocysteine, thus producing methionine and N^5, N^{10} dimethylglycine betaine (DMG) (McGregor et al., 2001). BHMT was shown to be

down-regulated in studies with mice with transient hyperhomocysteinemia caused by inhibition of BHMT in vivo (Collinsova et al., 2006). The same direction of regulation of BHMT in the methionine metabolism pathway was observed in our study in the experimental `MET´ diet. There are studies showing initial rate kinetics (Awad et al., 1983), ligand binding (Castro et al., 2004), and the crystal structure of the human BHMT- S-(δ-carboxybutyl)-DL-homocysteine (CBHcy) complex (Evans et al., 2002). These studies also confirmed the association of inhibition of betaine-homocysteine *S*-methyltransferase (BHMT) and hyperhomocysteine in human. Adenosylmethionine decarboxylase, AMD1, is an enzyme which catalyzes the conversion of *S*-adenosyl methionine to *S*-adenosylmethioninamine and contributes to the metabolism of componetes of the one-carbon cycle. AMD1 functions as a key enzyme in the biosynthesis of the polyamines spermidine and spermine (Nishimura et al., 2002) and plays an important physiological role in coordinating the availability of putrescine and decarboxylated AdoMet (Shirahata and Pegg, 1985).

The canonical pathway analysis revealed the IGF-1 Signaling pathway to be the highest significantly regulated pathway at 63 dpc; the expression of 8 genes (SHC1, FOXO1, YWHAH, IRS1, IGFBP3, IGFBP5, MAP2K1, PRKAG1) was down-regulated in this pathway. IGF-1 is a key growth factor during fetal development (Javaid et al., 2004) and may be involved in both normal and abnormal fetal growth. The stimulation of IGF-1 synthesis during normal pregnancy may be associated with an increase in GH production by the placenta. Thus, lower maternal and umbilical cord serum IGF-1 concentrations are associated with low birth weights of the newborns (Gómez, 2006). We confirmed the down-regulation of three IGF-1 signaling pathway genes (IGFBP3, IGFBP5 and SHC1) by real-time PCR. IGFBP3 and IGFBP5 are high affinity IGF binding proteins which are also involved in IGF-1 regulation, and it is important to include the IGF-independent properties, particularly those of IGFBP3 that may be involved in the osteoblastic differentiation observed in human bone marrow stromal cell cultures (Gómez, 2006). SHC1 is an important component in a mitogenic signal transduction pathway in which insulin; IGF-1 and EGF are involved (Sasaoka et al., 1994). Those genes were also show involved in effect of methionine parental nutrition and insulin-like growth factor 1 on methylation

events in rats (Yoshida et al., 1997: Resnick et al., 1986) and play a critical role in the ability of growth factors to promote normal development (Waly et al., 2004).

At 91 dpc the canonical pathways analysis revealed IGF-1 signaling pathway as the significant pathway. In it we found 31 genes regulated (PRKACB, CTGF, PDPK1, KRAS, YWHAQ, PTK2, AKT1, JUN, PIK3CG, SOS1, IGF1R, CSNK2A1, MRAS, RPS6KB2, AKT3, PIK3R2, RASA1, IGFBP6, PIK3C2A, RRAS, GRB2, YWHAZ, RAC1, IGFBP5, PIK3R3, RRAS2, PRKCI, PRKAG2, PRKCH, ELK1, PRKAR1A), most of which were up-regulated. The genes AAK1, SMAD5, MAP3K7 and IGFBP5 from the IGF-1 signaling pathway were selected for validation via quatitative real-time PCR in 24 individual animals at 91 dpc. AAK1 is a serine/threonine kinase that is thought to coordinate the recruitment of AP-2 to receptors containing tyrosine-based internalization motifs by phosphorylating the micro2 subunit. AAK1 functions at multiple steps of the endosomal pathway by regulating transferrin internalization and its rapid recycling back to the plasma membrane from early/sorting endosome (Henderson and Conner. 2007; Sorensen and Conner. 2008). SMAD5 is a mediator of the BMP signaling pathway; this protein is essential for the initiation of the definitive hematopoiesis (McReynolds et al., 2007), and can also transduce the inhibitory signal of TGF-beta1 on proliferation of hematopoietic progenitors derived from human bone marrow (Liu et al., 2003). Transforming growth factor-beta (TGF-beta) proteins regulate cell functions and have key roles in development and carcinogenesis in human (Derynck and Zhang, 2003). TAK1 or MAP3K7 is a member of the MAPKKK family and is activated by various cytokines, including transforming growth factor-β family ligands and interleukin-1 (IL-1). TAK1 is part of the transforming growth factor- β signaling pathways in mammalian cells (Kishimoto et al., 2000; Yamaguchi et al., 1995; Shirakabe et al., 1997).

Prenatal maternal diet affected the metabolic and health status in offspring of human (Symonds and Gardner, 2006) and mice (Cooney et. al., 2002). In particular, it was shown that methyl supplements have strong effects on DNA methylation and phenotype and are likely to affect long-term health. By canonical pathways analysis of postnatal stage we found IGF-1, SOCS3 and MAPK3 regulated due to gestation diets, i.e. showing down

regulation in case of maternal `MET´ diet. These are involved in IL-22 signaling, rhoA signaling, Fcγ receptor-mediated, phagocytosis in macrophages and monocytes, inositol phosphate metabolism and all 3 genes are included in growth hormone signaling. Insulin-like growth factor-1(IGF-1) is important anabolic hormone that regulates metabolic processes including protein synthesis in almost all tissues throughout the lifespan of mammals.

5.2.2 IPA pathway analysis to identification of gene functions in PI

The effects maternal nutrition during pregnancy in terms of balance of methionine supplementation on genes expression during fetal and postnatal life in PI were show differing weights of the fetuses demonstrated that maternal `MET´ treatment affects the phenotype of the offspring at 35, 91 dpc and postnatal ($p<0.05$). Moreover hepatic expression profiling provided large list of affected genes and canonical pathway including methionine metabolism pathway and IGF-1 signaling pathway at 63 and 91 dpc. Several studies examined the effects of methionine supplementation on gene expression, all indicating that an intake of methyl supplement starting at fertilization throughout pregnancy can have a profound impact upon gene expression (Zeisel, 2006). However, the results are inconsistent in terms of the differential expression due to maternal nutrition at post-implantation embryonic stage (Reik and Walter, 2001; Robertson and Wolffe, 2000) at early fetal stage in humans (Razin and Shemer, 1995), rats (Tsujiuchi et al., 1999), mice and cows (Dean et al., 2001; Wolff et al., 1998; Cooney et al., 2002). However, we could not describe on ambiguously trends of affects on differing weights of the fetuses and expression profiles clusters (Figure 4) that were separated into three district clusters according to stage but not into mother and diet, that may be due to influenced of environmental factor higher more than influence in the role of nutrition on the epigenetic regulation.

Ingenuity Pathway Analysis showed that important canonical pathway influenced by the gestation diets include methionine metabolism pathway at 91 dpc and IGF-1 signaling pathway at both 63 and 91 dpc in PI. Growth factor signaling categories pathway was regulated at 63, 91 dpc and postnatal stage. The canonical pathways were consistently affected and exhibit overlapping genes in all prenatal stages cover some amino acid

metabolic pathways and some signaling pathways including IGF-1 signaling. The pathways key found from our work were the role of methionine metabolism, IGF-1 signaling pathway and growth factor signaling in nutrition-mediated gene expression exhibited a strong association with growth development. These findings suggest that `MET´ diet acts as regulator of gene expression of metabolic genes in early fetus development that might be affected to growth development in 63, 91 dpc and postnatal stages. Wu et al. (2004) showed animal studies that both maternal under nutrition and over nutrition reduce placental fetal blood flows and stunt fetal growth. Impaired placental syntheses of nitric oxide (a major vasodilator and angiogenesis factor) and polyamines (key regulators of DNA and protein synthesis) may provide a unified explanation for intrauterine growth retardation in response to the 2 extremes of nutritional problems with the same pregnancy outcome. There is growing evidence that maternal nutritional status can alter the epigenetic state (stable alterations of gene expression through DNA methylation and histone modifications) of the fetal genome. This may provide a molecular mechanism for the impact of maternal nutrition on both fetal programming and genomic imprinting.

3 genes (PFN, PAK and Rho) revealed in differentially expressed gene list to be the highest significantly regulated genes at 35 dpc; PFN or Profilin is an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton. It is found in all eukaryotic organisms in most cells. Profilin is important for spatially and temporally controlled growth of actin microfilaments, which is an essential process in cellular locomotion and cell shape changes. This restructuring of the actin cytoskeleton is essential for processes such as organ development, wound healing, and the hunting down of infectious intruders by cells of the immune system. Profilin also binds sequences rich in the amino acid proline in diverse proteins. While most profilin in the cell is bound to actin, profilins have over 50 different binding partners. Many of those are related to actin regulation, but profilin also seems to be involved in activities in the nucleus such as mRNA splicing (Di Nardo et al. 2000). For Rho and PAK we found related and explained by Daniels and Bokoch (1999) that the mechanisms by which Rho family GTPases (Rho, Rac and Cdc42) regulate coordinated changes to the actin cytoskeleton are being elucidated. This review will focus on the current evidence that the p21-activated kinases (PAKs) are

involved in regulating some of the diverse cytoskeletal changes induced by Rac and Cdc42. PAKs have been shown to be required for processes including neurite formation and axonal guidance, development of cell polarity and motile responses. Signaling molecules interacting with PAKs that might contribute to the regulation of such processes have recently been identified.

The canonical pathways analysis revealed the IGF-1 Signaling pathway to be the highest significantly regulated pathway at 63 dpc; the expression of candidate genes (IGFBP3, IGFBP5, IGF2R) was up-regulated in this pathway due to methionine supplemented parental nutrition. IGF-1 is a key growth factor during fetal development (Javaid et al., 2004) and may be involved in both normal and abnormal fetal growth. The stimulation of IGF-1 synthesis during normal pregnancy may be associated with an increase in GH production by the placenta. Thus, lower maternal and umbilical cord serum IGF-1 concentrations are associated with low birth weights of the newborns (Gómez, 2006). We confirmed the up-regulation of three genes (IGFBP3, IGFBP5 and IGF2R) by real-time PCR. IGFBP3 and IGFBP5 are high affinity IGF binding proteins which are also involved in IGF regulation and it is important to include the IGF-independent properties, particularly those of IGFBP3 that may be involved in the osteoblastic differentiation observed in human bone marrow stromal cell cultures (Gómez, 2006).

At 91 dpc, DNMT1 and BHMT regulate homocysteine levels in the liver and reaction is metabolic source of dimethylglycine and is linked to the homocysteine metabolism as we described about in DL breed that we also found. MTAP (5'-methylthioadenosine phosphorylase) deficiency could be related to methionine-dependent growth. MTAP is a key enzyme in the methionine and adenine salvage pathways.in mammals (Berasain et al., 2004). Hellerbrand et al. (2006) detected a CpG island proximal to the transcription start of the MTAP promoter (at -461 to -441) and promoter-hypermethylation is causing functional relevant down regulation of methylthioadenosine phosphorylase (MTAP) expression in hepatocellular carcinoma. The ratio of SAM/SAH serves as an index of transmethylation potential. Transmethylation reactions have been shown to be compromised because of a decreased SAM/SAH ratio, particularly when SAH levels are elevated (Caudill et al., 2001;

Stam et al., 2004). It might be expected that the SAM/SAH ratio could be altered due to changes in activity of SAM-dependent methyltransferases, particularly BHMT, DNMT1 and MTAP.

At postnatal stage, we found FOXO1, BCL2 and MAFG. Forkhead box 01(FOXO1; forkhead in rhabdomyosarcoma, FKHR) is a key transcription factor that regulates the cell cycle and apoptosis, and therefore is considered to be involved in cell transformation and tumorigenesis. Emerging evidence indicates that protein levels of FOXO1 are under dual regulation by Ak-mediated phosphorylation and Skp2-mediated ubiquitination. (Huang and Tindall, 2006) Expression of FOXO1 in soft tissue sarcoma (STS) and its correlation with clinicopathological factors and prognostic significance were evaluated (Zhang et al., 2009). BCL-2 (B-cell lymphoma 2) is the founding member of the BCL-2 family of apoptosis regulator proteins encoded by the BCL2 gene. BCL-2 derives its name from B-cell lymphoma 2 (Reed and Pellecchia, 2005).

A straightforward mechanism for eliciting transcriptional repression would be to simply block the DNA binding site for activators. Such passive repression is often mediated by transcription factors that lack an intrinsic repressor activity. MAFG is a bidirectional regulator of transcription, a repressor in its homodimeric state but an activator when heterodimerized with p45. Here, we report that MAFG is conjugated to SUMO-2/3 in vivo. To clarify the possible physiological role(s) for sumoylation in regulating MAFG activity, we evaluated mutant and wild-type MAFG in transgenic mice and cultured cells. Whereas sumoylation-deficient MAFG activated p45-dependent transcription normally and did not affect heterodimer activity, repression by the sumoylation-deficient MAFG mutant was severely compromised in vivo. Furthermore, the SUMO-dependent repression activity of MAFG was sensitive to histone deacetylase inhibition. Thus, repression by MAFG is not achieved through simple passive repression by competing for the activator binding site but requires sumoylation, which then mediates transcriptional repression through recruitment of a repressor complex containing histone deacetylase activity (Motohashi et al., 2006).

5.3 SAM SAH measurements and DNA-methylation patterns of genes differentially regulated at foetal stages and at peripubertal age due to breed and gestation diet

The ratio of SAM/SAH serves as an index of transmethylation and remethylation potential (Loehrer et al., 2001). The main physiological function of this methionine metabolism pathway (Figure 7B) is probably the maintenance of adequate amounts of SAM, which not only plays a key role in the enzymatic regulation of homocysteine metabolising reactions, but is also the main methyl donor in numerous enzymatic *trans*-methylation reactions, which lead to the formation of SAH (Chiang et al., 1996). SAH acts as a competitive inhibitor of these SAM-dependent *trans*-methylation reactions and its affinity to most methyltransferases is greater than the affinity of the substrate SAM (Finkelstein, 1990). Increases in SAH concentration relative to SAM will therefore result in inhibition of *trans*-methylation reactions. Indeed, the ratio of SAM/SAH is crucial in the regulation of enzymatic *trans*-methylation reactions (Finkelstein, 1990; McKeever et. al., 1995) and a decrease of the ratio may inhibit *trans*-methylation reactions in various tissues (McKeever et al., 1995) thereby potentially affecting biosynthesis of a wide range of endogenous compounds such as proteins, hormones, phospholipids, neurotransmitters, DNA and RNA (Mathews and Holde, 1990).

Depending on the diets the SAM/SAH ration in sows and fetuses are at least partly opposite. The hypothesis is that if the SAM/SAH ratio in sows was decreased, it might be associated with increased SAM/SAH ratio in fetuses because of the imballanced absorbation or supply of the fetuses with SAH and SAH respectively. SAM/SAH ratio of fetus at 35 dpc was decreased in `MET´ and might be associated with inhibition of transmethylation reactions in various tissues (McKeever et al., 1995; Caudill et al. 2001; Stam et al., 2004; Castro et al., 2003). We selected BHMT, DNMT1 and MTAP for validation of differential expression that are involved in methionine pathway and showed down regulation. These genes encode enzymes of the one-carbon metabolisms that is key to DNA methylation; however the regualtion of these genes itself may be regulated due to inhibition of transmethylation reactions (Waterland et al., 2006).

In DL reduced SAM/SAH ratios at 35 dpc in `MET´ compared to CON is associated with reduced global DNA methylation. This is in line with the inhibitory effect of SAH on transmethyltion reactions leading to lower DNA-N-methyladenine or 5-methylcytosine. In 63 and 91 dpc, elevated ratios of SAM/SAH lead to an increased methylation levels of genomic DNA in `MET´ and therefore to the potentially detrimental alterations of IGF-1 signaling pathway both in 63 and 91 dpc and observed effects on growth hormone signaling pathway of postnatal stage. The regulation of expression of genes of the IGF pathway by DNA methylation is established (Waterland, 2006).

The analyses indicate an impact of the maternal gestation diet on global DNA methylation as well as the level of methylation of single CpG sites in selected differentially expressed genes observed in the offspring. However, the effects vary depending on ontogenetic stages and breed. Thus it is not possible to deduce a general trend of the effects of the maternal gestation diets on the methylation status. In order to gets a more comprehensive picture of the role of diet induced modulation of DNA methylation at various genes we aim to apply whole genome bisulfite sequencing analyses using next generation sequencing.

In conclusion, the results in these studies suggest that supplemented methyl in gestation diets affect hepatic genes expression at prenatal and postnatal stages and that vary dependent on developmental prenatal stage. Whether these changes reflect or lead to disturbances of any of the many supplementation important trans-methylation reactions requires further investigation. For example the specific gene DNA methylation may be more describe in mechanism of methylation reactions on developmental prenatal stage.

5.4 Implications and outlook

The project produced comprehensive lists of genes that were differentially expressed due to maternal gestation diets at three prenatal and postnatal stages in two breeds differing in carcass and meat quality traits and known to exhibit different hepatic expression profiles of fetuses and adults (Ponsuksili et al., 2007). The study comprises a valuable model for nutritional programming in humans, since pigs and humans share similarity in metabolism, physiology, anatomy and genome (Guilloteau et al., 2010; Lunney, 2007). The

experimental data complement existing findings from rodent models and epidemiological human data.

The aim of this paragraph is to provide a synthesis of the experiment, to sum up the major findings, and to highlight implications for further research.

Ontogenetic stage

Maternal gestation diets showed an acute effect on the offspring's hepatic transcriptome in fetal stages, when direct impact of the nutritional supply on the fetuses can be expected. However, the project also demonstrated long-term, delayed effects on the hepatic expression profiles of the offspring. In the field of fetal/nutritional programming often the term `persistent´ is used. The observed modulations of the transcriptome were not persistent in the sense that particular transcripts and/or pathways are permanently affected along the ontogenetic development. The experiment indicated a large effect of the ontogenetic stage on the diet-dependent shifts of expression levels.

Breed

Breed has a major effect regarding the consequences of feeding different maternal gestation diets. In terms of expression profiles, levels of global DNA methylation, gene-specific DNA methylation, and the adult phenotype breed differences were obvious. The impact of the maternal gestation diets were partly opposite in the two breeds.

Mother

Statistical analyses revealed that the effect of mother on expression profiles and phenotypes was large. Cluster analyses revealed that expression profiles of fetal liver were cluster first according to the ontogenetic stage, second according to the mother (even relationship among mothers is reflected by the clustering), and only in third instance according to the alternative maternal gestation diets.

Sex

Differential expression and phenotypic differences due to sex were not prominent in the experiment.

Modulation of the transcriptome

Though there is no permanent modulation of the expression level of particular genes from early fetal to peripubertal stage, the experiments indicated that mainly energy-sensing pathways, the methionine pathway, and molecular routes involved in growth and growth factor signaling were affected.

DNA methylation

The gestation diet modulates the methyl metabolism and the DNA methylation machinery in a complex fashion depending on ontogenetic stage and breed. (1) Shifts of the global DNA methylation, (2) regulation of genes encoding enzymes of the 1-carbon-cycle, and (3) variation of the SAM and SAH concentrations in liver among animals originated from either group of maternal gestation diets, together with the observed changes of the transcriptome and the phenotypes, promote the role of the epigenetic mechanism of DNA methylation as the basis of the molecular memory associated with “programming”. However, the effects vary depending on ontogenetic stages and breed. Thus it is not possible to deduce a general trend of the effects of the maternal gestation diets on the methylation status. Sofar, there is no obvious association between global DNA methylation and gene-specific DNA methylation. In order to get a more comprehensive picture of the role of diet induced modulation of DNA methylation at various genes the levels of DNA-methylation of CpG sites in the promoter region of differentially expressed genes should be determined. A most comprehensive insight could be obtained by whole genome bisulfite sequencing analyses using next generation sequencing.

Prenatal and adult phenotype

The experiment showed effects of the maternal gestation diets on intrauterine growth. At adult stage only slight but significant phenotypic effects on meat quality were observed. Liver plays a central role in the regulation of the metabolic status, partitioning of nutrients,

providing fuel to other tissues and expenditure of energy. Either the determination of metabolites in blood or liver (phenotypes more closely to the tissue used for expression profiling) or vice versa expression profiling of muscle, which is the tissue that is of major importance for the traits recorded, could provide a more obvious link of expression pattern and phenotype. The consistent finding of effects of gestation diets on meat colour in DL might be due to differences of the ultra structural properties of the muscle, i.e. muscle fiber traits, which are determined prenatal. This implies that the effects recorded at adulthood reflect acute effects of the gestation diets on the myogenesis of the offspring. Analyses of muscle fiber traits and expression of myogenic factors at prenatal stages will provide more insight into the association of prenatal modulation of expression of muscle tissue and adult changes of muscle properties. Accordingly, the study should be extended by performing real-time PCR of genes encoding myogenic factors and MyHC isoforms in prenatal and adult muscle (Wimmers et al., 2008).

The bioinformatics analysis of the data should be completed and deepened in terms of comparisons of expression patterns between breeds and between stages in order to obtain the diet-dependent differences of the changes of expression profiles that occur along ontogenetic development. Furthermore, the analyses will be extended in particular towards expression profiling of muscle tissue and towards gene-specific and genome-wide DNA methylation analysis.

A follow-on project would benefit from a design were a larger number of litters per experimental unit (diet×stage) is used, due to the immense effect of mother, whereas to total number of microarray hybridizations per experimental unit with a minimum of six seems adequate.

6. Summary

The impact of maternal gestation diets on gene expression profiles and phenotypes of offspring was shown experimentally and in epidemiological studies in model animals and humans. The study aimed at demonstrating foetal programming in pigs, listing genes sensitive to foetal programming, and evidencing the role of DNA-methylation in this phenomenon.

The whole experiment used 59 sows of the breeds German Landrace (DL) and Pietrain (Pi) that were fed methionine supplemented (`MET´) vs. control (`CON´) gestation diets. The `MET´-diet was supplemented with methionine and five co-factors (Vit B6, Vit B12, folate, betaine, choline) of the 1-carbon-cycle, in order to modulate the process of DNA-methylation.

Expression profiling of liver was done of fetal samples of three prenatal stages (35 dpc, 63 dpc, 91 dpc), and offspring at peripubertal age (150 dpn). In total 79, 81, and 74 fetuses at the respective stages were obtained and foetal samples were monitored for diet-dependent differential gene expression by using Affymetrix micorarrays (design: 3 sows (=litters) per breed, per stage, per diet; 2 pools of 6 animals each per litter). Moreover, for the breeds DL and Pi 96 and 56 piglets were obtained from 13 and 10 sows respectively. The offspring was born and raised at the research farms of the FBN Dummerstorf and the Faculty of Agricultural and Environmental Sciences of the University of Rostock, and its performance tested at the performance test station Jürgenstorf (Hybridschweinezuchtverband Nord/Ost e.V.). Traits related to growth performance were recorded. The pigs were slaughtered at the abattoir of the FBN Dummerstorf and carcass and meat quality data were collected according to the guidelines of the Zentralverband der Deutschen Schweineproduktion e.V. (ZDS, 2004). Animals were slaughtered at a mean age of 158 days. Hepatic RNA was used for expression profiling via Affymetrix microarrays using 12 animals of each breed, Pi and DL. Expression levels obtained from 78 microarrays were normalized using GC-RMA and log2 transformed to obtain genes differentially regulated at $p<0.05$ due to the gestational diet using a mixed model analysis within breed and stage, taking into account a nested design model for the mother within the diet: $Y = \mu + diet + mother\ (diet)$. Phenotypic data were analyzed using the Proc `Mixed´ of the SAS statistic software package applying a mixed model taking into account the random effect of the mother and the fixed effects of

gestation diet, gender, breed, RYR1 genotype and batch. Weight at slaughter and age at slaughter were considered for carcass and meat quality traits. For ordinal/nominal traits Proc `glimmix´ and Proc `freq´ were used. Aiming at the evaluation of DNA-methylation as a force driving differential expression, global DNA-methylation was measured. DNA samples from fetuses (35 dpc (n=74), 63 dpc (n=77), 91 dpc (n=79)) were isolated for measurement of global DNA methylation. The S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations were measured in liver samples from 17 sows (35 dpc: 5 sows, 63 dpc: 6 sows, 91 dpc: 6 sows) and pools of fetal livers representing both diet groups and all three ontogenetic stages by HPLC analysis (Kloor et. al., 2000).

The hepatic expression profiles revealed transcripts differentially expressed in DL (2055, 641, 2475 and 327 transcripts) and PI (618, 1539, 1592 and 112 transcripts) between the gestation diet groups at 35 dpc, 63 dpc, 91 dpc and at the postnatal stage, respectively. The Ingenuity Pathway Analysis was used to analyze the functional annotation of the significantly differentially expressed genes and pathway enrichment was calculated using a Fisher's exact test with a significance level of 0.05. The pathway categories that were consistently affected comprised growth factor signaling, cellular growth, proliferation development, intercellular and second messenger signaling, amino acid metabolism, apoptosis and nuclear receptor signaling. The important canonical pathways in both breeds included the methionine metabolism pathway, the IGF-1 signaling pathway and the growth hormone signaling Pathway. Differential expression between 'CON' and 'MET' was validated via qRT PCR for 11 genes from DL (BHMT, DNMT1, AMD1, IGFBP3, IGFBP5, SHC1, AAK1, SMAD5, MAP3K7, IGF1, SOCS3) and 12 genes from PI (PFN, Rho, PAK, IGFBP3, IGFBP5, IGF2R, BHMT, DNMT1, MTAP, FOXO1, BCL2, MAFG) all of which were selected from relevant canonical pathways.

Maternal gestation diets showed an acute effect on the offspring's hepatic transcriptome in fetal stages, when direct impact of the nutritional supply on the fetuses can be expected. However, the project also demonstrated long-term, delayed effects on the hepatic expression profiles of the offspring. The experiments indicated that mainly energy-sensing pathways and molecular routes involved in growth and growth factor signaling were affected.

The experiment showed effects of the maternal gestation diets on intrauterine growth. In DL, fetuses of the `MET´ group had lower body weights (P<0.001) (35 dpc: `CON´ (4.30±0.06), `MET´ (3.40±0.04); 63 dpc: `CON´ (165.60±26.87), `MET´ (156.70±23.90); 91 dpc: `CON´ (684.40±119.15), `MET´ (643.20±101.71)). However, weights of DL offspring at the peripubertal stage were not significantly different ('CON' (115.10±0.11) and 'MET' (114.70±0.22)). In PI offspring, weights of fetuses were affected (P<0.05) by the gestation diets at 35 dpc ('CON' (3.70±0.46), 'MET' (4.10±0.48)), at 91 dpc ('CON' (574.40±162.40, 'MET' (690.10±127.30)) and at the postnatal stage ('CON' (98,40±0.25), 'MET' (89.60±0.21)); differences observed at 63 dpc ('CON' (145.70±25.71), 'MET' (134.60±21.99)) were not significant.

The traits recorded for offspring of differentially fed gilts provide insight into the animals' growth performance, body and carcass composition and meat quality traits. According to the statistical evaluation, gestation diets had significant effects only on traits related to meat colour; in particular lightness and yellowness of the meat was affected by the diets fed to the mothers of the animals. Separate data analyses for each breed confirm significant effects of the gestation diets on meat color in DL (OPTO: `CON´ (68.94±1.15), `MET´ (72.92±1.19)). The interaction `diet×breed´ also showed significant differences in meat color (p<0.05). Interestingly, significant diet effects were found in the breed DL. Furthermore, there were differences in cold carcass weight in DL depending on the gestation diets. There are only subtle differences between the gestation diet groups within each breed. Effects of maternal gestation diets on prenatal growth were opposite in DL and PI. Postnatally, the nominal differences between gestation diet groups within breeds indicate a trend towards higher body weight before weaning and carcass weight in DL in `MET´, but in `CON´ in PI. Also for leanness and meat quality traits mostly opposite trends were observed in both breeds.

Global methylation of liver DNA was determined in 230 fetuses of the control and treatment group at all three stages in both breeds. Levels of DNA methylation differed significantly due to treatment ($p < 0.05$; `CON´ > `MET´), breed ($p < 0.0001$; PI > DL) and

prenatal stage ($p < 0.0001$; 91 dpc > 35 dpc > 63 dpc). The SAM/SAH ratios of sows were higher at all pregnancy stages in `CON´ compared to `MET´ in the breed PI, but differences between diet groups were not significant. In DL, the SAM/SAH ratio was significantly higher in `CON´ than in `MET´ at 91 days of pregnancy; the difference was attributable to a significant lower SAH concentration in liver tissue. In DL, at 35 dpc `CON´ and at 63 dpc `MET´ had nominal higher mean SAM/SAH ratios. Pooled tissues of fetuses revealed no significant differences.

The study delivered comprehensive lists of genes that were differentially expressed due to maternal gestation diets at three prenatal and one postnatal stage. Breed has a major effect regarding the consequences of feeding different maternal gestation diets. In terms of expression profiles, level of global DNA methylation, and the adult phenotype, breed differences were obvious. The impact of the maternal gestation diets was partly opposite in the two breeds. Both breeds analyzed differ in carcass and meat quality traits and are known to exhibit different hepatic expression profiles of fetuses and adults. Consequently, the breeds show different responses to the gestation diets. The gestation diet modulates the methyl metabolism and the DNA methylation machinery in a complex fashion depending on ontogenetic stage and breed. (1) Shifts of the global DNA methylation, (2) regulation of genes encoding enzymes of the 1-carbon-cycle, and (3) variation of the SAM and SAH concentrations in liver between the diet groups originated in both breeds. Together with the observed changes of the transcriptome and the phenotypes, this promotes the role of the epigenetic mechanism of DNA methylation as the basis of the molecular memory associated with “programming”.

7. Zusammenfassung

Der Einfluss der maternalen Ernährung während der Schwangerschaft auf die Genexpressionsprofile und die Phänotypen der Nachkommen wurde experimentell und in epidemiologischen Studien in Modelltieren und Menschen nachgewiesen. Diese Studie zielt darauf ab, fetale Programmierung in Schweinen nachzuweisen, Gene, die sensitiv auf fetale Programmierung reagieren, aufzulisten und die Rolle der DNA-Methylierung in diesem Phänomen zu belegen.

In dem Experiment wurden insgesamt 59 Sauen der Rassen Deutsche Landrasse (DL) und Pietrain (PI) verwendet, denen entweder eine mit Methionin angereicherte (`MET´) oder eine Kontrolldiät (`CON´) gefüttert wurde. Die `MET´-Diät war mit Methionin und fünf Cofaktoren des 1-Kohlenstoff-Zyklus (Vit. B6, Vit B12, Folat, Betain und Cholin) angereichert, um den Prozess der DNA-Methylation zu modulieren. Expressionsprofilanalysen der Leber wurden an Proben von Feten dreier pränataler Stadien (35, 63 und 91 dpc) und von Nachkommen im peripubertalen Alter (150 dpn) durchgeführt. Insgesamt wurden je 79, 81 und 74 Feten in den einzelnen Stadien gewonnen und die fetalen Proben wurden mittels Affymetrix Microarrays auf Diät-abhängig differentielle Genexpression hin überprüft (Design: 3 Sauen (=Würfe) pro Rasse, pro Stadium, pro Diät; 2 Pools aus je 6 Tieren pro Wurf). Weiterhin wurden Nachkommen der beiden Rassen (DL=96, PI=56) von je 13 (DL) und 10 (PI) Muttersauen gewonnen. Die Nachkommen wurden in den Forschungsfarmen des FBN Dummerstorf und der Agrar- und Umweltwissenschaftlichen Fakultät der Universität Rostock geboren und aufgezogen; ihre Wachstumsmerkmale wurden in der Landesprüfungsanstalt Jürgenstorf (Hybridschweinezuchtverband Nord/Ost e.V.) vermessen und aufgezeichnet. Die Schweine wurden im Schlachthaus des FBN Dummerstorf geschlachtet und Schlachtkörper- und Fleischqualitätsdaten wurden entsprechend der Richtlinien des Zentralverbands der Deutschen Schweineproduktion e.V. (ZDS, 2004) erhoben. Die Tiere wurden durchschnittlich im Alter von 158 Tagen geschlachtet. Für die Expressionsprofilanalyse mittels Affymetrix Microarrays wurde Leber-RNA von je 12 Tieren der Rassen PI und DL verwendet. Expressionslevel von 78 Microarrays wurden mittels GC-RMA normalisiert und log2-transformiert, um Gene zu bestimmen, die mit $p<0,05$ aufgrund der Diät während

der Trächtigkeit differentiell reguliert waren. Dazu wurde eine Gemischte-Modell-Analyse innerhalb von Rasse und Stadium durchgeführt, die ein geschachteltes Design-Modell der Mütter innerhalb der Diät annimmt: $Y = \mu + Diät + Mutter\ (Diät)$. Phänotyp-Daten wurden mit Hilfe des Proc 'Mixed' des SAS Statistik-Software-Pakets analysiert, wobei gemischte Modelle zur Anwendung kamen, die die Mutter als zufälligen Effekt und die Einflüsse der Diät während der Trächtigkeit, das Geschlecht, die Rasse, den RYR1-Genotyp und die Gruppe als fixe Effekte behandelten. Gewicht und Alter zum Zeitpunkt der Schlachtung wurden für Schlachtkörper- und Fleischqualitätsmerkmale berücksichtigt. Für ordinale/nominale Merkmale wurden die Proc 'glimmix' und 'freq' verwendet. Um die DNA-Methylation als eine treibende Kraft der differentiellen Expression zu evaluieren, wurde die globale DNA-Methylation gemessen. DNA wurde aus fetalen Proben (35 dpc (n=74), 63 dpc (n=77), 91 dpc (n=79)) isoliert und zur Messung der globalen DNA-Methylation verwendet. Die Konzentrationen von S-Adenosyl-Methionin (SAM) und S-Adenosyl-Homocystein (SAH) wurden in Leberproben von 17 Sauen (35 dpc: 5 Sauen, 63 dpc: 6 Sauen, 91 dpc: 6 Sauen) und Pools aus fetalen Lebern, die beide Diätgruppen und alle drei ontogenetischen Stadien repräsentierten, mittels HPLC-Analyse (Kloor et. al., 2000) gemessen.

Die hepatischen Expressionsprofile zeigten differentiell exprimierte Transkripte in DL (2055, 641, 2475 und 327 Transkripte) und PI (618, 1539, 1592 und 112 Transkripte) zwischen den Diätgruppen (jeweils zu den Zeitpunkten 35 dpc, 63 dpc, 91 dpc und in dem postnatalen Stadium). Ingenuity Pathway Analysis wurde benutzt, um die funktionelle Annotation der signifikant differentiell exprimierten Gene zu analysieren; die Signalweganreicherung wurde mittels eines exakten Tests nach Fischer mit einem Signifikanzlevel von 0,05 berechnet. Die konsistent betroffenen Signalwegskategorien umfassten Wachstumsfaktor-Signalwege, zelluläres Wachstum, Proliferationsentwicklung, interzelluläre und Second-Messenger-Signalwege, Aminosäuremetabolismus, Apoptose und Zellkernrezepter-Signalwege. Die wichtigsten kanonischen Signalwege in beiden Rassen beinhalteten den Methionin-Metabolismus-Signalweg, den IGF-1-Signalweg und den Wachstumshormonsignalweg. Differentielle Expression zwischen `CON´ und `MET´ wurde mittels qRT-PCR für 11 Gene in DL (BHMT, DNMT1, AMD1, IGFBP3, IGFBP5,

SHC1, AAK1, SMAD5, MAP3K7, IGF1, SOCS3) und 12 Gene in PI (PFN, Rho, PAK, IGFBP3, IGFBP5, IGF2R, BHMT, DNMT1, MTAP, FOXO1, BCL2, MAFG) verifiziert, die alle relevanten kanonischen Signalwegen entnommen wurden.

Die maternale Diät während der Trächtigkeit zeigte einen akkuten Effekt auf das Leber-Transkriptom der Nachkommen in fetalen Stadien, in denen eine direkte Wirkung auf die Nährstoffversorgung der Feten erwartet werden kann. Das Projekt wies allerdings auch verzögerte Langzeiteffekte auf die hepatischen Expressionsprofile der Nachkommenschaft nach. Die Experimente deuten darauf hin, dass hauptsächlich stark Energie abhängige Stoffwechselwege und molekulare Prozesse des Wachstums und der Wachstumsfaktor-Signalwege beeinflusst wurden.
Das Experiment zeigte Effekte der maternalen Diät während der Trächtigkeit auf das intrauterine Wachstum. Die DL-Feten der `MET´-Gruppe hatten geringere Körpergewichte als die der `CON´-Gruppe ($P<0.001$) (35 dpc: `CON´ (4.30±0.06), `MET´ (3.40±0.04); 63 dpc: `CON´ (165.60±26.87), `MET´ (156.70±23.90); 91 dpc: `CON´ (684.40±119.15), `MET´ (643.20±101.71)). Im peripubertalen Stadium waren die Gewichte der DL-Nachkommen jedoch nicht signifikant verschieden (`CON´ (115.10±0.11) und `MET´ (114.70±0.22)). Bei den PI-Nachkommen beeinflusste die Diät während der Trächtigkeit das Gewicht zu den fetalen Zeitpunkten 35 dpc (`CON´ (3.70±0.46), `MET´ (4.10±0.48)) und 91 dpc ('CON' (574.40±162.40, `MET´ (690.10±127.30)) und im postnatalen Stadium (`CON´ (98,40±0.25), `MET´ (89.60±0.21)); zum Zeitpunkt 63 dpc gemessene Unterschiede (`CON´ (145.70±25.71), `MET´ (134.60±21.99)) waren nicht signifikant.

Die aufgezeichneten Daten der Nachkommenschaft der unterschiedlich gefütterten Jungsauen gewähren Einblick in die Wachstumsleistung, die Körper- und Schlachtkörperzusammensetzung und die Fleischqualitätsmerkmale der Tiere. Laut der statistischen Auswertung hatte die Diät während der Trächtigkeit signifikante Effekte lediglich auf Merkmale der Fleischfarbe; insbesondere die Helligkeit und der Gelbanteil des Fleisches waren von den Diäten, die den Müttern der Tiere gefüttert worden waren, betroffen. Separate Analysen der Daten für jede Rasse bestätigten die signifikanten Effekte der Trächtigkeitsdiäten auf Fleischfarbe in DL (OPTO: `CON´ (68.94±1.15), `MET´

(72.92±1.19)). Die Interaktion 'Diät×Rasse' zeigte ebenfalls einen signifikanten Effekt auf die Fleischfarbe (p<0.05). Interessanterweise fanden wir signifikante Diätffekte in der Rasse DL. Weiterhin gibt es Unterschiede im Schlachtgewicht der DL in Abhängigkeit von den Trächtigkeitsdiäten. Es gibt nur geringfügige Unterschiede zwischen den Diätgruppen einer Rasse. Effekte der maternalen Diät während der Trächtigkeit auf pränatales Wachstum wirkten sich in DL und PI gegensätzlich aus. Postnatal zeigen die nominalen Unterschiede zwischen den Diätgruppen innerhalb einer Rasse einen Trend zu höherem Körpergewicht vor der Entwöhnung und höherem Schlachtkörpergewicht in der Gruppe `MET´ für DL, aber in der Gruppe `CON´ für PI. Außerdem wurden hauptsächlich entgegengesetzte Trends für Magerkeit und Fleischqualitätsmerkmale in beiden Rassen beobachtet.

Globale Methylation der Leber-DNA wurde in 230 Feten aller drei Stadien der Kontroll- und der Behandlungsgruppe in beiden Rassen bestimmt. Der Grad der DNA-Methylierung variierte signifikant aufgrund der Behandlung ($p < 0.05$; `CON´ > `MET´), der Rasse ($p<0.0001$; PI > DL) und des pränatalen Stadiums ($p < 0.0001$; 91 dpc > 35 dpc > 63 dpc). Der SAM/SAH-Quotient war bei PI-Sauen aller Trächtigkeitsstadien höher in der 'CON'- als in der `MET´-Gruppe, aber Unterschiede zwischen den Diätgruppen waren nicht signifikant. In DL war der SAM/SAH-Quotient in der `CON´-Gruppe am Tag 91 der Trächtigkeit signifikant höher als in der `MET´-Gruppe; dieser Unterschied konnte auf die signifikant niedrigere SAH-Konzentration im Lebergewebe zurückgeführt werden. In DL hatte `CON´ zum Zeitpunkt 35 dpc und `MET´ zum Zeitpunkt 63 dpc nominal höhere durchschnittliche SAM/SAH-Quotienten. Gepoolte Gewebe von Feten zeigten keine signifikanten Unterschiede. Die Studie liefert umfangreiche Listen von Genen, die aufgrund der maternalen Trächtigkeitsdiäten an drei pränatalen und einem postnatalen Stadium differentiell exprimiert werden. Die Rasse hat einen deutlichen Effekt, was die Konsequenzen der unterschiedlichen maternalen Trächtigkeitsdiät betrifft. Offensichtliche Rassenunterschiede gab es bezüglich der Expressionsprofile, des Grades der globalen DNA-Methylation und des adulten Phänotyps. Der Einfluss der maternalen Trächtigkeitsdiät war in den beiden Rassen teilweise entgegengesetzt. Die beiden untersuchten Rassen unterscheiden sich in Schlachtkörper- und Fleischqualitätsmerkmalen und weisen unterschiedliche hepatische Expressionsprofile in Feten und adulten Tieren auf.

Als Konsequenz daraus zeigen die Rassen unterschiedliche Reaktionen auf die Trächtigkeitsdiäten. Die Diät während der Trächtigkeit moduliert den Methylmetabolismus und die DNA-Methylationsmaschinerie in komplexer Weise, in Abhängigkeit von ontogenetischem Stadium und Rasse. (1) Änderungen der globalen DNA-Methylation, (2) Regulation von Genen, die für Enzyme des 1-Kohlenstoff-Zyklus codieren, und (3) Variation der SAM- und SAH-Konzentrationen in der Leber zwischen den Diätgruppen zeigten sich in beiden Rassen. Zusammen mit den beobachteten Veränderungen des Transkriptoms und des Phänotyps belegt dies die Rolle des epigenetischen Mechanismus der DNA-Methylation als basalen Teil des molekularen Gedächtnisses, das mit der 'Programmierung' assoziiert ist.

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