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Hepatic and muscular transcriptomic responses of porcine progeny to gestational diets varying in protein content

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Michael Oster

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Schmalkalden

Referent: Prof. Dr. Klaus Wimmers

Korreferent: Prof. Dr. Peter Stehle

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Abstract

Inadequate maternal protein supply during gestation represents an environmental factor that is known to affect physiological signaling pathways and impacts the phenotype of the progeny in animal models and in humans with long-term consequences for growth, function and structure of various tissues. The provoked intrauterine adaptive responses are termed 'Fetal Programming'. Hypothesising that the progeny's transcriptome is persistently altered by maternal diets, we used a porcine model to monitor the longitudinal expression changes in liver and muscle tissue to identify pathways relevant to fetal initiation of postnatal growth and development. Throughout the whole pregnancy nulliparous German landrace sows were fed one of three isoenergetic diets differing in their protein:carbohydrate ratio, resulting in a low protein diet (LP: 6.5 % crude protein), a high protein diet (HP: 30 % crude protein) and an adequate protein diet (AP: 12.1 % crude protein). All progeny was nursed by foster sows that received a standard diet. Postweaning, the progeny was fed standard diets *ad libitum*. The progeny's liver (n = 192) and muscle tissue (*M. longissimus dorsi*; n = 72) were collected at 94 days post conception (dpc) and 1, 28, and 188 days post natum (dpn) for expression profiling. The analyses included comparisons between dietary groups within ontogenetic stages as well as comparisons between ontogenetic stages within dietary groups to separate diet-specific transcriptional changes and maturation processes. An *in utero* exposure to adverse gestational protein diets revealed a 'Programming' of the progeny's genome in a diet-, tissue- and stage-dependent manner. Transcriptional alterations during pre- and postnatal development differed considerably among dietary groups, particularly for genes related to energy metabolism, lipid metabolism, cell cycle regulation, organismal and cellular growth, and glucocorticoid receptor signaling. No single gene was found differentially expressed between the groups along all examined stages. The transcriptional responses in both HP and LP progeny were interpreted as the molecular equivalent to developmental plasticity which accounts for adaptation and maintenance of the organismal phenotype and affects signaling pathways related to energy utilisation. Obviously, the 'Fetal Programming' of the genome warrants adaptation processes regarding to compensatory growth, probably at the expense of a predisposition for metabolic disturbances up to adult stages.

Kurzbeschreibung

Ungünstige Umwelteinflüsse während der fetalen Entwicklung bewirken eine intrauterine adaptive Antwort mit langfristigen Konsequenzen auf Wachstum, Funktion und Struktur verschiedener Gewebe der Nachkommen. Diese Zusammenhänge werden als 'Fetale Programmierung' bezeichnet. So stellt eine unangepasste maternale Proteinversorgung während der Gestation einen Umweltfaktor dar, der sowohl physiologische Signalwege beeinflusst als auch phänotypische Anpassungen bei Nachkommen von Mensch und Tier hervorruft. In der vorliegenden Arbeit wurde die Hypothese einer persistenten Transkriptomregulation der Nachkommen in Abhängigkeit von der mütterlichen Diät in einem porcinen Model bearbeitet. Ziel war es, molekulare Signalwege und Mechanismen mit Relevanz zur fetalen Initiierung von postnatalem Wachstum und Entwicklung zu identifizieren. Jungsauen der Deutschen Landrasse wurden während der gesamten Trächtigkeit mit einer von drei isoenergetischen Diäten ernährt, die sich in ihrem Protein:Kohlenhydrat-Verhältnis unterschieden. Die experimentellen Gruppen wurden als Niedrigproteindiät (LP, 6% Rohprotein, Protein:Kohlenhydrat 1:10), Hochproteindiät (HP, 30% Rohprotein, Protein:Kohlenhydrat 1:1,3) und adäquate Proteindiät (AP, 12% Rohprotein, Protein:Kohlenhydrat 1:5) bezeichnet. Die Nachkommen wurden von Ammensauen gesäugt, welche mit Standardfutter ernährt wurden. Nach dem Absetzen erhielten die Nachkommen Standardfutter *ad libitum*. Um Genexpressionsprofile abzubilden, wurde der Nachkommenschaft Leber ($n = 192$) und Muskelgewebe (*M. longissimus dorsi*; $n = 72$) am 94. Tag der Trächtigkeit (dpc) sowie am 1., 28., und 188. Lebenstag (dpn) entnommen. Durch das longitudinale Studiendesign konnten sowohl Vergleiche zwischen Diätgruppen innerhalb der ontogenetischen Stadien als auch Vergleiche zwischen ontogenetischen Stadien innerhalb der einzelnen Diätgruppen vorgenommen werden. Auf diese Weise war es möglich, Diät-spezifische transkriptionelle Änderungen von physiologischen Reifungsprozessen zu unterscheiden. Die *in utero* Exposition zu nicht bedarfsgerechten maternalen Proteindiäten zeigte eine 'Programmierung' des Genoms der Nachkommenschaft in Abhängigkeit von Diät, Gewebe und ontogenetischem Stadium. Die Diät-abhängigen transkriptionellen Regulationen in den Nachkommen umfassten Änderungen des Lipidmetabolismus, der Zellzyklusregulation, des Energiemetabolismus, des organismischen

und zellulären Wachstums sowie des Glukokortikoidrezeptor Signalweges. Es wurden keine Gene gefunden, die in allen untersuchten ontogenetischen Stadien eine differentielle Auslenkung zwischen den Diätgruppen aufwiesen. Die transkriptionelle Auslenkung in den HP und LP Nachkommen wurde als molekulares Äquivalent einer Entwicklungsplastizität interpretiert, welche für die Adaptation und den Erhalt des rassetypischen Phänotyps sorgt und daneben Signalwege der Energieausnutzung beeinflusst. Offensichtlich garantiert die 'Fetale Programmierung' des Genoms ein rassespezifisches Wachstum, welches mit einer Prädisposition für metabolische Störungen bis zum Erwachsenenalter einhergeht.

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1 Introduction

Intrauterine growth retardation (IUGR), i.e. impaired prenatal growth and development, is a major concern in animal breeding due to its negative impact on postnatal performance and health. Particularly pregnancy diets have been shown to impact the progeny's phenotype. In various rat models a relationship between maternal protein intake and the progeny's birth weight was shown. This included long-term effects on immune status, growth, body composition and associated parameters like decreased glucose tolerance, insulin resistance and alterations in energy metabolism ('Thrifty Phenotype'). In this context it is of high interest that lower birth weight piglets ($<$1.2 kg) from otherwise normal litters reveal lower daily gains, higher body fat contents, lower muscle mass and higher drip losses postnatally. The causal mechanisms are termed 'Fetal Programming' and are not well understood.

1.1 The Project FEPROeXPRESS - 'Fetal Programming' due to dietary protein deficit and excess in pigs

Based on this background the DFG project PRÄNATS and the adjacent BMBF FUGATOplus project FEPROeXPRESS were designed. On the one hand it was investigated whether isoenergetic gestational diets containing different protein amounts affect growth, body composition, plasma metabolites, and hormones in porcine offspring at prenatal and postnatal stages. On the other hand, diet-dependent effects of the offspring's transcriptome (microarray analysis) and proteome (2D-Electrophoresis and peptide mass fingerprinting using MALDI-TOF-MS) were analysed. Diet-dependent and tissue-specific signals and metabolic routes were identified to explain altered productivity traits and metabolic statuses. Molecules found to be diet-dependently regulated were partially verified by independent methods (quantitative real-time PCR, Western blot). Moreover, diet-dependent excursions of candidate molecules (involved in the somatotropic axis, protein metabolism and energy utilisation) were investigated in plasma and tissues (ELISA, Western ligand blot). Further, *in vitro* enzyme activity assays were conducted. Furthermore, it was determined whether epigenetic processes (e.g. DNA

methylation, detected via bisulfide pyrosequencing) have an impact on long-term transcriptional alterations and metabolic parameters due to the dietary intervention during fetal time.

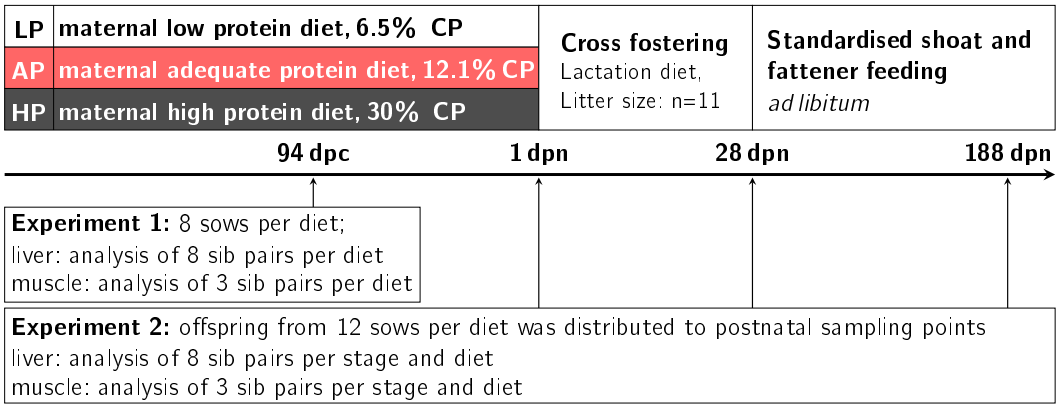

Figure 1.1: Experimental design. Throughout the whole pregnancy nulliparous German landrace sows were fed one of three isoenergetic diets differing in their protein:carbohydrate ratio, resulting in a low protein diet (LP: 6.5 % crude protein), a high protein diet (HP: 30 % crude protein) and an adequate protein diet (AP: 12.1 % crude protein). All the progeny was nursed by foster sows that received a standard diet. Postweaning, the progeny was fed standard diets *ad libitum*. The progeny's liver (n = 192) and muscle tissue (*M. longissimus dorsi*; n = 72) were collected at 94 days post conception (dpc) and 1, 28, and 188 days post natum (dpn). CP - crude protein.

Corresponding to the metabolic body weight nulliparous German Landrace sows were fed one of three isoenergetic diets (≈13.7 MJ ME / kg on average) differing in their protein:carbohydrate ratio throughout the whole pregnancy (Figure 1.1), resulting in a low protein diet (LP: 6.5 % crude protein, protein:carbohydrate ratio 1:10), a high protein diet (HP: 30 % crude protein, protein:carbohydrate ratio 1:1.3) and an adequate protein diet (AP: 12.1 % crude protein, protein:carbohydrate ratio 1:5). The progeny's liver (n = 192) and muscle tissue (*M. longissimus dorsi*; n = 72) were collected at 94 days post conception (dpc) and 1, 28, and 188 days post natum (dpn) for expression profiling using genome-wide GeneChip® Porcine Genome Arrays. At 94 dpc fetuses derived from a subset of eight sows per dietary group were sampled (Experiment 1). In a second experiment (Experiment 2) the offspring of 12 litters per dietary group was distributed over the three postnatal time points (1, 28, 188 dpn). Litter size during suckling was standardized to 11 piglets per sow. The progeny of all dietary groups was nursed by foster sows that received a standard diet. Postweaning, the progeny was fed standard diets *ad libitum*. For the microarray analyses, sib pairs balanced for sex (all stages) and discordant for weight (light and heavy piglets within one litter; stages 94 dpc and 1 dpn only) were chosen per stage and diet.

The project was supposed to provide basic data regarding the fetal initiation of postnatal growth, development, metabolic health, and mechanism of growth retardation due to early nutritional challenges in a porcine model. The analyses open new insights into the interaction between genotype and maternal nutritional supply in early life and investigated the susceptibility of adaptation and growth causing variation in birth weight. In this thesis, diet-dependent effects focussing the transcriptome of fetuses and offspring are presented. This includes the identification of molecular routes with a possible relevance regarding the fetal initiation of postnatal growth performance and metabolic impairments.

1.2 State of research

About 20 years ago, a relationship between poor fetal growth and subsequent development of type-2 diabetes in later life was pointed out by British epidemiologists [1]. However, markers used to define poor fetal growth rates are usually proxy indices, based on records of birth weight, length, abdominal and head circumferences and various relative values [2]. Considering these phenotype observations, it is impossible to estimate whether an organism attained its full growth potential *in utero* [2]. There are multiple reasons for a lowered weight at birth, including maternal (e.g. nutrient supply, body weight, body length), placental (e.g. hormone production, umbilical blood flow rate, placenta size) and fetal factors (e.g. genotype, fetal growth factors, metabolic rate). Time point and duration of these factors also determine the occurring consequences (e.g. symmetrical or asymmetrical growth retardation). Nevertheless, a strong correlation between low birth weight on the one hand and metabolic impairments on the other hand is well documented. Various epidemiological data in human confirmed a link between poor early growth and susceptibility to impaired glucose tolerance [3], abnormal lipid metabolism, hypertension, and type-2 diabetes [1], adiposity [4], and cardiovascular diseases [5, 6]. Furthermore, studies on twins (both monozygotic and dizygotic) who are discordant for diabetes revealed that the diabetic twin had lower birth weight [7, 8].

1.2.1 Development of the 'Thrifty Phenotype Hypothesis'

There is evidence, provided by studies on individuals who were exposed to famine *in utero*, that fetal growth rate and metabolic health in later life depend on the maternal nutrient status one was exposed to during fetal time. Both men and women subjected to the 'Dutch hunger winter' (1944-45) *in utero* had a lowered birth weight and showed increased plasma glucose

concentrations at adulthood [9]. Furthermore, depending on time point and duration when an individual is exposed to famine during fetal time, insulin concentrations were found to be altered at adulthood. These observations indicated an association between poor maternal nutrient supply and insulin resistance and diabetes in the offspring in later life, despite some potential maternal confounders (e.g. exposure to cold, mental stress, exhausting activity) may act on the children's growth rates as well [10].
The collected epidemiological observations were summarized to generate the 'Thrifty Phenotype Hypothesis' [11]. This hypothesis proposed that early malnutrition provokes physiological compensations promoting early survival at the expense of later health [12]. Therefore, metabolic health has got a potent environmental basis. When an organism is malnourished during fetal development and therefore lowered in birth weight or organ weight it will develop strategies to maximize its chances for postnatal survival - the so called 'Predictive Adaptive Response Hypothesis' [13]. Due to developmental plasticity, interactions between genotype and environment lead to adaptation processes (e.g. in gene expression) and ultimately to a modified phenotype [14]. It was proposed that malnourished fetuses will graduate its organs in a hierarchical order to provide them with micro- and macronutrients, e.g. the brain grows at the expense of liver and skeletal muscle tissue [15]. The exposure to malnutrition in early life might lead further to adaptations of the transcriptome [16], proteome [17] and metabolome [18] to increase its chances of survival in the face of adverse environmental conditions. However, these processes become inappropriate or will contribute to metabolic impairments (e.g. obesity) if there is a mismatch between functional capability set during fetal development and functional requirements of the postnatal environment, e.g. prenatal nutrient restriction and postnatal adequate nutrition [19].

1.2.2 Concept of 'Fetal Programming'

Offspring undergoing switches between pre- and postnatal nutrient statuses was shown to be prone to accelerated growth performance [20, 21] and metabolic disturbances like hypertension [22, 23], hypertriglyceridemia [24, 25] or impaired insulin metabolism [26, 27]. This relationship was named 'Developmental Origins of Health and Diseases' (DOHaD) [28]. Once only a result of epidemiological studies, the 'Thrifty Phenotype Hypothesis' now is supported by several findings originating from both human studies and animal models. The causal processes and mechanisms leading to phenotype adaptations due to adverse prenatal environmental conditions are named 'Fetal Programming' (Figure 1.2). According to scientific discussions in

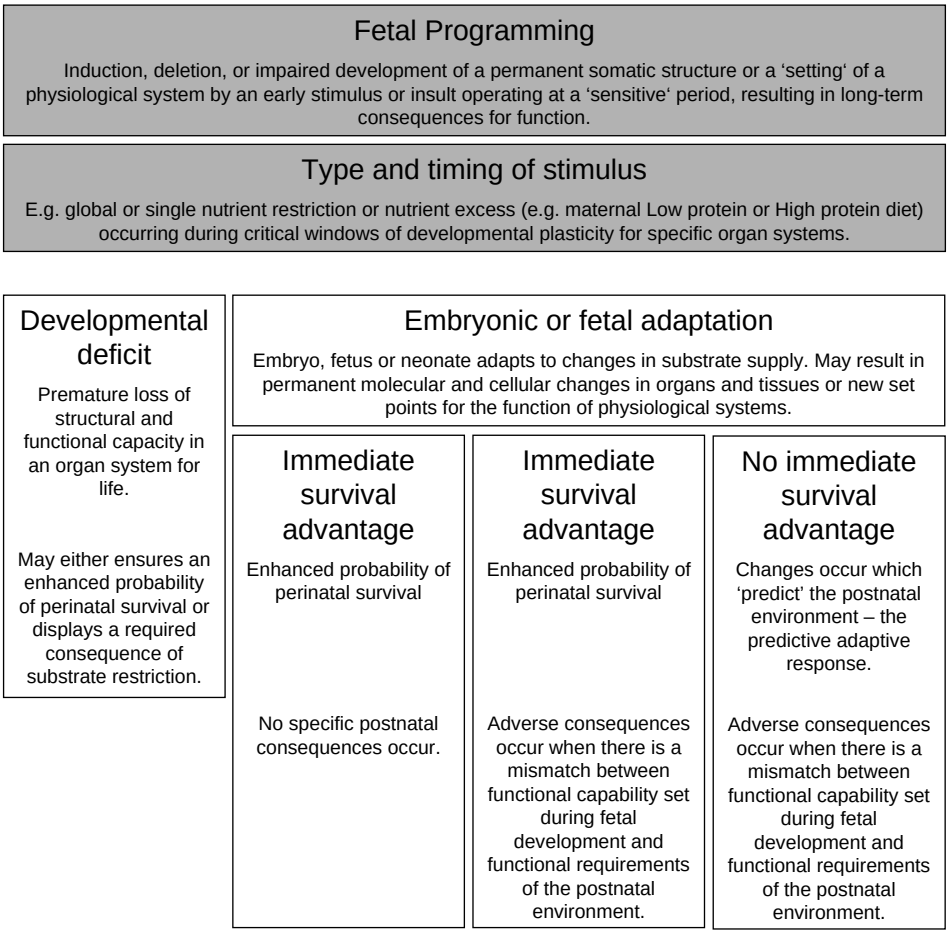

Figure 1.2: A summary of the current mechanisms considered to underlie the 'Developmental Origin of Health and Disease' (DOHaD) concept. (Adapted from McMillen & Robinson, 2005 [19])

the early nineties of the last century, 'Fetal Programming' is described as induction, deletion or impaired development of a permanent somatic structure [29]. This approach was extended by McMillen and Robinson, who stated, that an early stimulus affects the physiological system in a sensitive period which may cumulate in long-term consequences for function and structure [19]. The basal molecular mechanisms underpinning these long-term programmed effects regarding phenotype modifications and metabolic excursions in later life are likely to be complex and multifactorial and include alterations in maternal hormone and metabolite levels [30, 31]. Due to developmental plasticity offspring's gene and protein expression patterns vary in response to

these maternal signals. Therefore, 'Fetal Programming' may include persistent regulations of the offspring's transcriptome leading to the formation of a molecular 'Dietary Memory'. However, dietary interventions failed to show persistent regulations of specific pathways, but revealed long-term modulations of gene expression [32, 33]. In the context of long-term transcriptional alterations, epigenetic processes like DNA methylation [34] and histone modification [35] might contribute to the control of gene expression. Therefore, both chromatin assembly as well as regulatory DNA sections might be influenced. It has been shown, that these mechanisms are also sensitive to environmental factors like early nutrition [36, 37]. The occurring different methylation pattern of CpG-dinucleotids might lead to a long-term control of either gene expression or gene inactivation. Notably, DNA methylation patterns are bequeathed via mitosis but remain reversible.

1.2.3 Questions regarding survival strategies of malnourished fetuses and offspring

Research focussing on the 'Fetal Programming' phenomenon primarily investigates the association between low birth weight and developing human adult diseases. Furthermore, impaired fetal growth and development impact postnatal performance and health, which is of high relevance in animal breeding [38]. Therefore, the composition of a maternal gestation diet is seen of important relevance in both preventive medicine and in farm animal breeding. As it was defined by McMillen and Robinson [19] 'Fetal Programming' occurs due to an early stimulus during a critical window of developmental plasticity, e.g. an adverse maternal dietary supply throughout pregnancy (Figure 1.2). The complex adaptive mechanisms between prenatal environmental clues and survival strategies of the fetuses and offspring raised a number of questions, which are investigated by scientific research groups worldwide:

- Which effects regarding transcriptome, proteome, and metabolome are revealed in dependence to various adverse maternal diets?
- What are the primary targets of 'Fetal Programming'?
- Which metabolic pathways and functional networks trigger the observed changes in phenotype?
- Which time periods have to be considered as critical windows of developmental plasticity due to maternal malnutrition?
- Are those effects species-specific?

1.2.4 Animal models investigating the 'Thrifty Phenotype Hypothesis'

To estimate the impact of non-genetical factors contributing to IUGR a large volume of animal work has been done. In feeding experiments investigating rodents and farm animals, the 'Thrifty Phenotype Hypothesis' was extensively tested. Scientific research in the past few years investigated dietary manipulations in rats, mice, sheep, cattle, and pigs. The analysed feeding regimes showed short-term as well as long-term effects caused by different maternal diets, including a maternal low protein diet, maternal high protein diet, maternal global restriction, and maternal high fat diet. The dietary manipulations were conducted during various pregestational, prenatal as well as postnatal time frames to analyse potential windows of developmental plasticity. Intensively investigated effects of dietary manipulations are displayed in Figure 1.3. The width of each time frame box ('Days before mating', 'Gestation', 'Lactation', and 'Postweaning') displays one hundred per cent of each species-specific developmental period. Notably, the diet-dependent effects described in this thesis are indicated by the red labeled experimental designs, displaying maternal low and high protein diets throughout gestation.

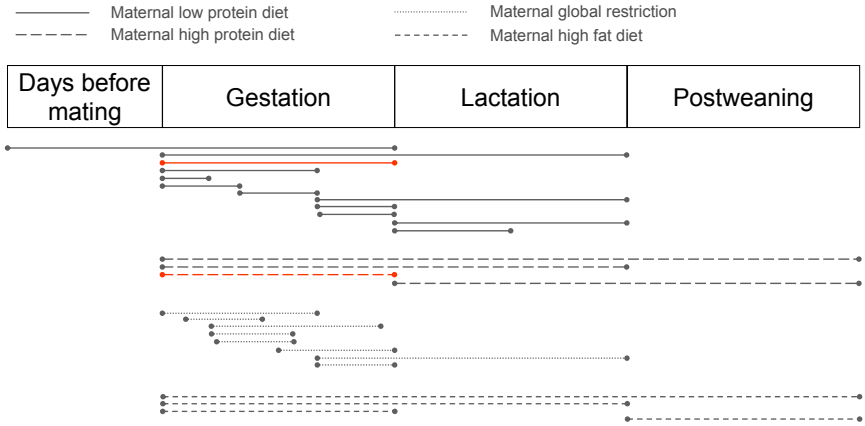

Figure 1.3: Relative experimental designs of different dietary interventions in mammals described in detail in Table 1.1. Taken from recent publications the exposure time to different maternal feeding regimes is shown, including maternal low protein diets, maternal high protein diets, maternal global restrictions, and maternal high fat diets. The species-dependent duration of gestation and lactation of rat, mouse, pig, sheep, and cattle were taken into account in a relative manner. In this thesis effects due to the red labeled experimental designs (maternal low and high protein diets throughout gestation) were described in a porcine model.

In the past few years various experimental data of rats, mice, sheep, cattle, and pigs supported the 'Thrifty Phenotype Hypothesis'. Thus, the proposed relationship between maternal diet,

early growth, gene expression, metabolic status and health performance in malnourished fetuses and offspring became evident. The various maternal dietary interventions visualized in Figure 1.3 were summarized with regard to prenatal and postnatal effects observed in offspring (Table 1.1). Because interactions between genotype and environment lead to adaptations in gene expression and ultimately to a modified phenotype [14], particular attention was paid to both organismal changes and diet-dependent mRNA regulation. Therefore, observed phenotype parameters like body weight, growth performance and body composition as well as effects on both single gene expression and signaling pathways were compiled. Furthermore, diet-dependent alterations of DNA methylation patterns, proteome profiles, and metabolite levels were taken into account.

Table 1.1: Effects observed in offspring due to maternal malnutrition during pregnancy and lactation in selected species The type of dietary manipulation, its exposure time as well as a distinction of diet-dependent effects related to organismal changes, gene expression and other observed modifications are displayed. * These cases had a modified nutrient supply both in control group and treated group before or after the time of intervention. Abbreviations: dpc - days post conception; dpn - days post natum

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	Gene expression	Other effects
pig				
6% vs 12% protein	0 dpc-term	$\Downarrow$ birth weight [39], $\Downarrow$ primary & secondary fibres (1 dpn, 28 dpn) [20], $\Downarrow$ number of adipocytes (1 dpn) [20], catch-up growth [20], $\Uparrow$ body fat (28 dpn) [20]	affected pathways in liver tissue: short-term and long-term alterations of cell cycle regulation, glucocorticoid receptor signaling, lipid metabolism (94 dpc - 188 dpn) [40]	
8.5% vs 14% protein	0-75 dpc	$\Downarrow$ birth weight [41]		
8.5% vs 14% protein	0 dpc-term	$\Downarrow$ birth weight [41]		
30% vs 12% protein	0 dpc-term	$\Downarrow$ fetal weight (94 dpc) [33], $\Downarrow$ birth weight [39], $\Downarrow$ number of adipocytes (1 dpn) [20], $\Downarrow$ body fat (1 dpn) [20]	affected pathways in liver tissue: short-term and long-term alterations of OXPHOS, AMPK signaling, mTOR signaling, glucocorticoid receptor signaling, lipid metabolism (94 dpc - 188 dpn) [33]	
sheep				
50% vs 100% maternal intake	0-95 dpc	$\Downarrow$ liver weight (male) (3 years) [42]	$\Downarrow$ GR, prolactin, IGF-2R, HGF, SOCS-3 in liver (male) (3 years) [42], $\Uparrow$ VDAC, Bax in liver (male) (3 years) [42]	
50% vs 100% maternal intake	28/30 - 78/80 dpc	$\Downarrow$ carcass weight (78 dpc) [43], $\Leftrightarrow$ birth weight [44], $\Uparrow$ liver weight (240 dpn) [45], $\Downarrow$ secondary myofibres (78 dpc) [43], $\Uparrow$ muscle fibre diameter (240 dpn) [45]	$\Uparrow$ AT1R and GR in kidney, liver, lung, adrenals (1 dpn) [44], $\Downarrow$ 11βHSD2 in kidney, adenals (1 dpn) [44], $\Uparrow$ hepatic PPARγ, PGC1α, HSD11B1, HSD11B2 (1 year) [24], $\Leftrightarrow$ hepatic GCK, GR, UCP-2, IR, IGF-1R, PRKAA2 (1 year) [24]	$\Downarrow$ activity of CPT1 in skeletal muscle (240 dpn) [45], $\Downarrow$ phosphorylation of mTOR and S6K in skeletal muscle (78 dpc) [43], $\Leftrightarrow$ activity of AMPK in skeletal muscle (78 dpc) [43], $\Uparrow$ intramuscular TAG (240 dpn) [45], $\Uparrow$ hepatic TAG (1 year) [24]

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	Gene expression	Other effects
70% vs 100% maternal intake	26-135 dpc	⇓ fetal weight of heart, pancreas, thymus, gut, kidney (135 dpc) [46], ⇔ liver weight (135 dpc) [46]		
cattle				
68% vs 100% maternal intake	31-125 dpc	⇓ carcass weight (125 dpc) [47]		⇓ phosphorylation of mTOR and S6 kinase in skeletal muscle (125 dpc) [47]
mouse				
8% vs 20% protein	0 dpc-term	⇓ birth weight [48, 49], catch-up growth (7 dpn) [49], ⇑ body weight (7-21 dpn) [49]	affected pathways in liver and skeletal muscle: oxidative phosphorylation, fatty acid metabolism, carbohydrate metabolism (1 dpn) [48]	
8% vs 20% protein	0 dpc-term + lactation	⇓ birth weight [49], ⇓ body weight (7-21 dpn) [49]		
40% vs 20% protein	0 dpc-term	⇓ body weight (3 dpn, 21 dpn) [50], ⇓ liver weight (3 dpn, 21 dpn) [50]	affected pathways in liver: acute response/complement system, GH/JAK/STAT/IGF signaling (3 dpn), protein/ fatty acid/ hexose/ pyruvate metabolism (21 dpn) [50]	
40% vs 20% protein	0 dpc-term + lactation	⇓ body weight (3 dpn, 21 dpn) [50], ⇓ liver weight (3 dpn) [50]	affected pathways in liver: acute response/complement system, GH/JAK/STAT/IGF signaling (3 dpn); protein/ fatty acid/ hexose/ pyruvate metabolism (21 dpn) [50]	
44% vs 25% protein	0 dpc-term + lactation			affected pathways in liver: amino acid degradation, carbohydrate and fatty acid metabolism, gluconeogenesis, citrate cycle, protein folding, secretion of proteins, (de)activation of transcription factors (14 dpn, 23 dpn) [51]
60% vs 12% fat	0 dpc-term + lactation	⇑ body weight (3-21 dpn) [52], ⇔ weights postweaning (35-140 dpn) [52]	⇓ DAT in hypothalamus, ⇑ DAT, MOR, PENK in nucleus accumbens, prefrontal cortex, VTA (126-168 dpn) [52]	hypomethylation in brain of DAT, MOR, PENK (126-168 dpn) [52]
rat				
0% vs 22% protein	1-10 dpn lactation	⇓ body weight (male) [53], ⇓ epididymal fat pad weights (male) (90 dpn) [53]		adipocytes: ⇓ tyrosine phosphorylation of IR, IRS-1 after insulin stimulation (male) (90 dpn) [53], ⇑ basal phosphorylation of IRS-2, Akt, mTOR (male) (90 dpn) [53], ⇑ basal glucose uptake (male) (90 dpn) [53], ⇑ GLUT4 in plasma membrane (male) (90 dpn) [53]
5% vs 15% protein	14.5 dpc-term	⇓ fetal weight (21.5 dpc) [54], ⇓ birth weight [55], ⇔ body weight (60 dpn) [55], ⇓ fetal β-cell mass (21.5 dpc) [54]		⇔ plasma insulin, glucose (60 dpn) [55], ⇓ pankreatic insulin content (60 dpn) [55], ⇔ glucose tolerance/utilisation/production (60 dpn) [55]
5% vs 20% protein	0 dpc-term	⇓ birth weight (1 dpn) [56]; ⇓ liver weight (1 dpn) [56]	⇓ hepatic IGF-1 (1 dpn) [56]	⇓ plasma IGF-1 (1 dpn) [56], ⇓ liver IGF-1 (1 dpn) [56]

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	Gene expression	Other effects
6% vs 18% protein*	14 days before mating-term (20% protein lactation)	⇓ body weight (21 dpn) [22]		⇑ systolic blood pressure (63 dpn, 147 dpn) [22]
8% vs 18% protein	0 dpc-term + lactation	⇓ pancreas weight (male) (175 dpn) [57], ⇓ β-cell mass (male) (175 dpn) [57]		⇓ hepatic mtDNA (male) (35 dpn) [57], ⇑ hepatic mtDNA (male) (105 dpn) [57], ⇓ muscle and pancreatic mtDNA (male) (175 dpn) [57]
8% vs 20% protein	0 dpc-term	⇓ fetal liver weight (21 dpc) [18], ⇔ fetal weight (21.5 dpc) [58], ⇓ birth weight [58, 59, 21], ⇓ birth weight (male) [60], ⇓ birth weight (female) [61], catch-up growth (12 dpn) [21], catch-up growth (male) (7 dpn) [60], catch-up growth (female) (7-14 dpn) [61], ⇓ body weight (3-21 dpn) [62], ⇓ islet vascularisation, β-cell proliferation, islet size (1 dpn) [58, 63]	⇓ hypothalamic CCND1, CDKN1a, DNMT1, DNMT3a, IGF-1 (1 dpn) [21], ⇑ CCND1 (male) (28 dpn) [64]; ⇓ PPARγ, SREBP-1c (male) (28 dpn) [64], affected pathways: mitochondrial biogenesis, function and metabolism in pancreatic islands (90 dpn) [59]	⇓ plasma IGF-I (21 dpc) [18], ⇑ plasma and hepatic 29-32kDa IGFBPs (21 dpc) [18], ⇑ plasma leptin (12 dpn) [21], ⇑ fasting glucose concentration (21 dpn) [61], ⇑ MnSOD (female) (21 dpn) [61], ⇔ plasma leptin, TAG, glucose, insulin (male) (22 dpn) [60], ⇑ proliferation rate of adipocytes (male) (28 dpn) [64], ⇓ insulin response to glucose (70 dpn) [65, 66], ⇑ ROS production (male) (90 dpn) [59], ⇑ plasma insulin (112 dpn) [67], ⇓ TAG (180 dpn) [68]
8% vs 20/21% protein	0 dpc-term + lactation	⇓ birth weight [68, 21, 30, 69, 70], ⇓ birth weight (female) [71], ⇓ birth weight (male) [60, 72], ⇓ β-cell mass with smaller islets (1-21 dpn) [69], ⇓ body weight (5-16 dpn) [21, 70], ⇓ body weight (male) (2-510 dpn) [60, 73], ⇓ organ growth (selective) (21 dpn) [74]	⇑ hypothalamic DNMT1 (12 dpn) [21], ⇓ pancreatic IGF-2 (19.5 dpc-14 dpn) [69], ⇑ hypothamamic NPY, AgRP (male) (22 dpn) [60], ⇑ hepatic ACC α, FAS (male) (26 dpn) [70], affected pathways in visceral adipose tissue: ⇓ inflammation, ⇑ carbohydrate metabolism, lipid metabolism, protein metabolism, adipocyte differentiation, angiogenesis, extracellular matrix remodeling (male) (130 dpn) [72], ⇔ GLUT4 (450 dpn) [75], ⇓ PKCζ in muscle (male) (450 dpn) [75]	⇑ pancreatic immunoreactivity of CCND1 (21.5 dpc, 14dpn, 17 dpn) [69], ⇑ pancreatic islet cell apoptosis (21.5 dpc-21 dpn) [69], ⇓ pancreatic insulin content (1 dpn) [69], ⇓ plasma leptin, plasma insulin (5 dpn, 12 dpn) [21], ⇑ glucose level (14 dpc) [30], ⇓ plasma TAG, glucose, insulin (22 dpn) [60], ⇓ serum insulin (male), NEFA (male) (26 dpn) [70], ⇑ serum glucose (male), serum TAG (male), hepatic TAG (male) (26 dpn) [70], ⇔ plasma insulin (112 dpn) [67], ⇓ islet blood flow (112 dpn) [67], ⇓ plasma cholesterol, HDL-cholesterol, TAG (180 dpn) [68], ⇔ glucose uptake in muscle (male) (450 dpn) [75], impaired insulin stimmulated glucose uptake in muscle (male) (450 dpn) [75], ⇑ fasting plasma glucose (male) (510 dpn), insulin concentration (male) (510 dpn) [73], ⇑ plasma glucose (male), plasma insulin after IVGTT (male) (510 dpn) [73], ⇑ plasma insulin after IVGTT (female) (630 dpn) [71], ⇓ PKCζ in muscle (female), p110-associated p85 in abdominal fat (female) (630 dpn) [71]

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	**Gene expression**	**Other effects**
8% vs 21% protein	14 dpc-term + lactation	⇓ birth weight [70], ⇓ body weight (1-26 dpn) [70]	⇑ hepatic ACCα and FAS (male) (26 dpn) [70]	⇓ serum insulin, NEFA [70], ⇑ serum TAG, hepatic TAG (male) (26 dpn) [70]
8% vs 20% protein	lactation			⇑ insulin sensitivity (female) (21 dpn) [61], ⇑ renal IR-β, IGF-1R, Akt1, Akt2, Sirt1, CAT, CuZn-SOD, GPx1 (female) (21 dpn) [61], ⇓ plasma cholesterol, HDL-cholesterol, TAG (180 dpn) [68]
8% vs 23% protein	lactation	⇓ body weight (up 180 dpn) [76], ⇓ visceral fat mass, total body fat (180 dpn) [76]		⇓ serum insulin, glucose (180 dpn) [76], ⇑ total adrenal catecholamine content, serum corticosterone (180 dpn) [76]
9% vs 18% protein	14 days before mating-term	⇑ fetal weight (20 dpc) [77], ⇓ fetal weight (21 dpc) [78], ⇓ organ weight (21 dpc) [78], asymetrical organ growth (kidney, heart) (21 dpc) [79], ⇓ birth weight [80, 81]	affected pathways: hepatic IGF-1 signaling, VEGF signaling, cell differentation, cytokine signaling (21 dpc) [16], ⇑ renal CDKN1 (21 dpc) [79]	proteome alterations in liver tissue: proteins associated with amino acid metabolism, mitochondrial function and cell motility (21 dpc) [16], ⇑ systolic blood pressure (28 dpn, 49 dpn, 63 dpn, 147 dpn) [22, 80, 77, 81], ⇑ GPDH activity (1 dpn) [77], hypermethylation hepatic DNA (21 dpc) [78]
9% vs 18% protein	0-4.25 dpc	⇓ birth weight (female) [82], ⇑ body weight (28-49 dpn) [82]	⇑ hepatic PEPCK (male), 11β-HSD1 (female) (20 dpc) [83], ⇔ hepatic IR, GR, GS, GLUT1, GLUT3 (20 dpc) [83]	⇑ systolic blood pressure (male) (28 dpn, 77 dpn) [82]
9% vs 18% protein*	days 0-7 (20% protein 8 dpc-postweaning)	⇔ body weight (30 dpn, 270 dpn, 540 dpn) [25]	⇓ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP (270 dpn) [25], ⇑ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP, PPARγ (540 dpn) [25], ⇓ PPARα, MCAD, IRS-2 (540 dpn) [25]	⇑ hepatic TAG (540 dpn) [25], ⇑ plasma TAG (540 dpn) [25], ⇑ plasma cholesterol (female) (540 dpn) [25]
9% vs 18% protein	0 dpc-term	⇓ birth weight [17, 23], ⇓ birth weight (male) [84], ⇔ birth weight [85], ⇔ birth weight (male) [86], catch-up growth (male) (30 dpn) [84], ⇓ nephron number (28 dpn, 140 dpn) [85], ⇓ glomeruli number (150 dpn) [23], ⇓ pancreas weight (male) (175 dpn) [57]	⇓ REN in kidney (1 dpn, 5 dpn) [23], ⇑ GR in kidney (1 dpn), lung (20 dpc, 84 dpn), hypothalamus (84 dpn) [17], ⇓ AT2R (female) (28 dpn) [85], ⇑ renal Na/K-ATPase a1/b1 (84 dpn) [17], ⇓ 11β-HSD2 in kidney (84 dpn) [17], ⇓ AT1R (140 dpn) [85], ⇑ AT2R (female) (140 dpn) [85], affected pathways in adipose tissue: carbohydrate metabolism, lipid metabolism, adipose tissue expressed molecules (CFD, AGT, Cav-1, RETN, SPARC, SREBP-1c, Transferrin) (male) (270 dpn) [84]	⇑ GR protein in kidney (20 dpc, 14-84 dpn), liver (14dpn, 84 dpn), lung (20 dpc, 14 dpn, 84 dpn) [17], ⇑ arterial & systolic blood pressure (male) (42 dpn, 130 dpn, 150 dpn) [87, 86, 23], ⇓ hepatic mtDNA (male) (35 dpn) [57], ⇑ hepatic mtDNA (male) (105 dpn) [57], ⇓ muscle and pancreas mtDNA (male) (175 dpn) [57]

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	Gene expression	Other effects
9% vs 18% protein*	0 dpc-term (19/20% protein lactation - postweaning)	⇓ fetal weight (female) (20 dpc) [88], ⇑ nephron number (20 dpc) [89], ⇔ birth weight [90], ⇓ nephron number (28 dpn) [89], ⇔ body weight (30 dpn, 270 dpn, 540 dpn)	⇑ hepatic DNMT1 (female) (18 dpc) [88], ⇑ hepatic 11β-HSD1 (male) (20 dpc) [83], ⇔ hepatic IR, GR, GS, GLUT1, GLUT3 (20 dpc) [83], ⇔ hepatic PPARα, PPARγ (21 dpc) [91], ⇑ hepatic PPARα (34 dpn) [91], ⇔ hepatic PPARγ (34 dpn) [91], ⇓ adipose PPARγ (34 dpn) [91], ⇑ hepatic GR, PPARα (34 dpn) [92], ⇓ hepatic DNMT1 (34 dpn) [93], ⇑ hepatic GR (34 dpn) [93], ⇓ TRβ1 (male) (84 dpn) [94], ⇑ c-Fos, iNOS, TNFα, NFκB, UCP-3 (84 dpn) [94], affected pathways in liver: response to ROS, steroid hormone respone, ion transport, developing process (84 dpn) [94], ⇓ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP (270 dpn) [25], ⇔ GAL, GAL2R (270 dpn, 540 dpn) [90], ⇑ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP, PPARγ (540 dpn) [25], ⇓ PPARα, MCAD, IRS-2 (540 dpn) [25]	⇑ hepatic CAT activity (20 dpc) [88], altered fatty acid compositions in liver, brain, lung, plasma and heart phospholipids (34 dpn) [95], ⇑ plasma TAG and NEFA (34 dpn) [91], ⇓ hepatic GR methylation, histone modifications (34 dpn) [92, 93], ⇓ hepatic PPARα methylation (34 dpn, 80 dpn) [92, 96], ⇑ systolic blood pressure (28 dpn, 130 dpn) [89], ⇑ hepatic TAG (540 dpn) [25], ⇑ plasma TAG (female) (540 dpn) [25], ⇑ plasma cholesterol (female) (540 dpn) [25], no impact of homocysteine [88], ⇓ hepatic PPARα, GR methylation of F1 (male) (80 dpn) and F2 animals (male) (80 dpn) [97]
9% vs 18% protein*	8-14 dpc (20% protein 0-7 dpc, 15 dpc - postweaning)	⇔ body weight (30 dpn, 270 dpn, 540 dpn) [25]	⇓ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP (270 dpn) [25], ⇑ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP, PPARγ (540 dpn) [25], ⇓ PPARα, MCAD, IRS-2 (540 dpn) [25]	⇑ hepatic TAG (540 dpn) [25], ⇑ plasma TAG (female) (540 dpn) [25]
9% vs 18% protein*	15 dpc-term (20% protein 0-14 dpc, lactation - postweaning)	⇔ body weight (30 dpn, 270 dpn, 540 dpn) [25]	⇓ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP (270 dpn) [25], ⇑ hepatic LPK, FAS, ACC1, SREBP-1c, ChREBP, PPARγ (540 dpn) [25], ⇓ PPARα, MCAD, IRS-2 (540 dpn) [25]	⇑ hepatic TAG (540 dpn) [25], ⇑ plasma TAG (male) (540 dpn) [25], ⇑ plasma cholesterol (male) (540 dpn) [25]
9% vs 22% protein*	14 days before mating-term (17% protein lactation - postweaning)	⇔ birth weight [98], ⇓ body weight (female) (133-154 dpn) [98], ⇓ fat pads (female) (175 dpn) [98]		⇓ insulin after oGTT (175 dpn) [98], ⇓ hepatic PEPCK activity (female) (175 dpn) [98]
40% vs 20% protein	0 dpc-term	⇓ body weight (male) (2 dpn) [99], ⇑ body weight (male) (19 dpn, 40dpn) [99], ⇑ body fat (male) (60 dpn) [99]		⇓ energy expenditure (male) (62 dpn) [99]
40% vs 20% protein	0 dpc-term + lactation + postweaning	⇓ body weight (male) (2 dpn) [99], ⇔ body weight (male) (19-60 dpn) [99], ⇑ body fat (male) (60 dpn) [99]		⇓ energy expenditure (male) (62 dpn) [99]
40% vs 20% protein	lactation + postweaning	⇑ body weight (male) (19-40 dpn) [99]		
19% vs 3% fat	0 dpc-term	⇓ muscle fibres (21 dpn) [100]	⇓ IR, PCNA in muscle (21 dpn) [100], ⇔ IGF-1, IGF-1R, PPARγ in muscle (21 dpn)[100]	

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	**Gene expression**	**Other effects**
19% vs 3% fat	0 dpc-term + lactation	⇓ muscle fibres (21 dpn), ⇑ intramuscular fat, adipocyte hypertrophy (21 dpn) [100], ⇔ liver mass (70 dpn) [101]	⇑ IGF-1, IGF-1R, PPARγ (21 dpn) [100], ⇑ hepatic PPARα (male), PPARδ (male), LRH (male), UCP-2, GLUT2 (male), SOD1, SOD2 (male), CAT (male), IGF-1R (male), IR (male) (70 dpn) [101], ⇓ hepatic PPARδ (female) (70 dpn) [101]	
19% vs 3% fat	0 dpc-term + lactation + postweaning	⇔ liver mass (70 dpn) [101]	⇑ hepatic PPARα (male), PPARδ (male), SOD1, SOD2 (male), LRH (male), UCP-2 (male), GPx1, GLUT2 (male), FAS (male), GCK, CAT, IRS-1, IR (male) (70 dpn) [101], ⇓ hepatic PPARδ (female), LRH (female), GLUT2 (female), IGF-1R (female), IRS-2 (female) (70 dpn) [101], ⇑ hepatic TAG (70 dpn) [101]	hepatic steatosis (70 dpn) [101]
23% vs 4% fat	0 dpc-term	⇔ birth weight [27], ⇑ body weight (female) (90 dpn) [27], ⇑ β-cell mass (female) (90 dpn) [27]	⇑ pancreatic NRF1 (female), PPARγ (male), ATP6 (male) (90 dpn) [27], ⇓ pancreatic GLUT-2 (female), UCP-2, GCK (female), MDH (female), ATP6 (female) (90 dpn) [27], ⇓ hepatic Tfam (female), ND4L (female), ATP6 (female), MDH (90 dpn) [27]	impaired insulin release (islets) (90 dpn) [27], ⇓ plasma insulin (female) (90 dpn) [27], ⇓ ATP (islets) (90 dpn) [27]
30% vs 100% maternal intake	0 dpc-term	⇓ body weight, liver weight (55 dpn) [26]	⇔ transcriptome muscle and fat (male) (55 dpn, 110 dpn) [26], affected hepatic pathways: carbohydrate/glucose metabolism, lipid metabolism, amino acid metabolism, protein turnover, apoptosis, mitochondrion/electron transport chain (male) (55 dpn, 110 dpn) [26]	⇑ obisity and altered insulin sensitivity (male) (110 dpn) [26], ⇑ body fat (110 dpn), plasma LDL (55 dpn), leptin, insulin (110 dpn) (male) [26]
50% vs 100% maternal intake	0 dpc-term	⇓ birth weight [27], catch-up growth (90 dpn) [27], ⇓ β-cell mass (90 dpn) [27]	⇑ pancreatic PGC1α, PPARγ (male), MDH (male) (90 dpn) [27], ⇓ pancreatic Tfam, ATP6, UCP-2 (male), GCK (male), MDH (female) (90 dpn) [27], ⇑ hepatic ND4L (male), COX1 (male) (90 dpn) [27]	impaired insulin release (islets) [27], ⇑ plasma cholesterol (female) [27], ⇑ mtDNA (islets) [27], ⇓ ATP (islets) (90 dpn) [27]
50% vs 100%maternal intake	11 dpc-term	⇓ birth weight [102], ⇓ body weight (100 dpn) [102]		⇓ plasma glucose, insulin (20 dpc) [102], ⇑ plasma glucose (100 dpn) [102], ⇓ insulin sensitivity (100 dpn) [102], ⇔ glucose uptake in liver, adipose tissue (100 dpn) [102]
50% vs 100%maternal intake	14.5/15 dpc-term	⇓ birth weight [103], ⇓ body weight (21.5 dpc) [54], ⇔ body weight (14.5 dpc, 21 dpn, 56 dpn) [55, 103], ⇓ β-cell mass (21.5 dpc, 1 dpn, 21 dpn) [54, 103]		⇓ pancreatic insulin content (21.5 dpc, 1 dpn, 21 dpn) [54, 103], ⇓ plasma insulin (56 dpn) [55], ⇑ pancreatic insulin content (56 dpn) [55], ⇔ glucose tolerance, utilisation, production (56 dpn) [55], ⇔ plasma glucose (56 dpn) [55]

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Table 1.1: Continued

Manipulation	Dietary intervention	Effects observed in offspring		
		Organismal changes	Gene expression	Other effects
50% vs 100%maternal intake	15 dpc-term + lactation	$\Downarrow$ β-cell mass and number (21 dpn, 252 dpn) [104], $\Uparrow$ β-cell proliferation (insufficient) (252 dpn) [104]		

In particular, the impact of the low protein model was investigated in various human and animal studies. It has been shown that maternal low protein intake during pregnancy is associated to low birth weight, and subsequently to disorders of metabolic health as well as body composition of the offspring [17, 19]. Interestingly, it has been demonstrated that a low protein diet fed to pregnant dams will lead to IUGR and reduced birth weight [22, 58, 73]. Much less is known about possible effects of gestational high protein diets on offspring development and later health. It has been previously shown in rats, mice, and pigs that maternal excessive protein intake during pregnancy is associated to IUGR, and results suggest that this is related to net energy deficiency in the gestating dam [39, 51, 99]. Interestingly, epidemiological studies in women show that high protein intakes during pregnancy can also result in fetal growth retardation [105, 106, 107]. Additionally, also high birth weight children are at risk of a range of health problems that have both immediate implications and long-term consequences [108, 109]. Taken together, experimental and epidemiological data of animals and humans confirm that the relationship between birth weight and adult metabolic impairments like obesity, insulin resistance, or hypertension, displays a parabola. As suggested by Desai et al., 2011, the optimal birth weight of an organism will be potentially specific to the life conditions of the individual mother, which will minimizes the potential of metabolic impairments [110].

1.2.5 Pig as an animal model

Because of their easy handling and short reproduction rate studies on 'Fetal Programming' were mainly done in rodent animal models. Pig studies also display a valuable model for 'Fetal Programming' in humans, since pigs and humans share similarities in metabolism, physiology, anatomy and genome [111, 112]. Furthermore, both humans and pigs are omnivorous nourished. Nevertheless, pigs are a litter bearing species, which does not reflect the human situation of usually only one child per pregnancy. Any litter effect might bias developmental processes which may cumulate in altered fetal growth or birth weight [113, 114]. Hence, the human pregnancy is rather mimicked by sheep or cattle, because ewes or cows give birth to usually

one to two offspring per pregnancy. Humans and sheep share a long gestation and their offspring is born with similar birth weight and pattern of organ development. Further, fetal and perinatal development are comparable to humans in terms of the rapid growth of adipose tissue. On the contrary, there are strong differences concerning the ruminant digestive system in comparison to the human monogastric omnivorous ones which might bias maternal dietary interventions. In summary, there are advantages and disadvantages by selecting farm animal models to approximate human conditions.

1.2.6 Experimental protein diets in a porcine model

Most studies on 'Fetal Programming' were focused on biochemical and transcriptional changes of selected candidate metabolic and/or signaling pathways and their related genes at particular developmental stages. Thus, there is only little knowledge about genes that are targets of 'Fetal Programming'. However, longitudinal holistic studies of the modulation of the offspring's transcriptome due to maternal dietary supply during pregnancy are scarce. Therefore, the use of high-throughput techniques like microarray analysis gives us the opportunity to get better insights into the diet-dependent gene regulation [33, 72, 94].
In order to contribute to a comprehensive inventory of genes and functional networks that are targets of nutritional programming initiated during fetal life, we applied whole-genome microarrays for expression profiling in a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages in a porcine model (Figure 1.1). On an isoenergetic basis, pregnant sows were fed either a gestational low protein diet (LP, 6.5 % crude protein), a high protein diet (HP, 30 % crude protein) or an adequate protein diet (AP, 12.1 % crude protein) to investigate the effects on hepatic and muscular gene expression in their fetuses and offspring. The experiment comprises a valuable model especially in terms of 'fetal' programming, because cross-fostering enabled assessment of solely the nutritional effects during gestation. The longitudinal holistic study design presented in this project is eligible to trace diet-dependent modulations of the offspring's transcriptome among ontogenesis.
Existing findings from rodent models and epidemiological human data were complemented by porcine studies. In particular, the low protein diet provides a model for prenatal undersupply and exposure to famine that regrettably still burdens a considerable proportion of the human population. Furthermore, the high protein diet displays a model for dietary recommendations to combat obesity in humans.
The differences of diet-dependent expression profiles were arranged in four manuscripts investi-

gating both adverse dietary treatments (HP vs. AP & LP vs. AP) in liver and skeletal muscle tissue. We demonstrated that the expression profiles showed considerable modulations during prenatal and postnatal stages as indicated as immediate and delayed transcriptional responses to the nutritional stimulus. The diet-dependent transcriptional alterations indicated different functional demands and replies of liver and skeletal muscle tissue in the experimental groups under identical postnatal nutrient conditions.

1.3 Aims

The overall objective of this thesis was to elucidate the fetal initiation of postnatal growth, development and metabolic health in non-rodent species. Mechanisms of growth retardation and subsequent compensatory processes due to early nutritional challenges were investigated on the transcriptional level. This included the identification of relevant molecular pathways in terms of growth, productivity traits, stress, and metabolic impairments in porcine progeny. The holistic expression analyses pointed out to which extent a maternal protein diet led to persistent regulations of the transcriptome. The expression patterns found in the dietary groups were associated to phenotype effects. Depending on maternal diet, ontogenetic stage and investigated tissue a general view of transcriptional regulations sensitive to 'Fetal Programming', in particular to IUGR, adaptation mechanisms and metabolic impairments, were deduced.

2 Publications

2.1 Summary of publications

This thesis deals with hepatic and muscular transcriptomic responses of porcine progeny to gestational diets varying in protein content. The resulting dietary comparisons (HP vs. AP and LP vs. AP, respectively) were processed separately in both analysed tissues as displayed in four manuscripts.

In liver tissue, the overall synthesis of the expression analysis indicates that maternal protein supply provokes a 'Programming' of the progeny's genome. The 'Programming' is characterized by the adjustment of energy sensing pathways, energy producing pathways (HP) as well as molecular signaling pathways related to cell cycle regulation, proliferation and maintenance (LP). Furthermore, stage-dependent regulations among the treatment groups reflect compensatory growth that is associated with the observed IUGR in both HP and LP progeny. However, the modulation of these pathways could indicate a predisposition for metabolic disorders in adulthood in both HP and LP offspring. The results have been reported in section 2.2, *Plos One*, 2011, **6(7)**, e21691 and in section 2.3, *BMC Genomics*, 2012, **13(1)**, 93.

In skeletal muscle tissue, mRNA abundances of both HP and LP fetuses and offspring revealed associations with the observed IUGR. The effects of 'Fetal Programming' due to inadequate maternal protein diets provoke transcriptional responses regarding to pathways related to growth and energy utilisation up to adult stage. In HP progeny the diet-dependent longitudinal shifts of transcriptional responses led to a minimization of differences at later stages. In contrast, the expression profiles in LP progeny indicate an adaptive response in terms of compensatory growth. It is characterized by a slight shift of body composition towards fat accumulation at the cost of muscle growth. This is in line with observations of the organismal phenotypes. Further details are described in section 2.4, *Plos One*, 2012, **7(4)**, e34519 and in section 2.5, *Physiol Genomics*, 2012, **44(16)**, 811-8.

2.2 A high protein diet during pregnancy affects hepatic gene expression of energy sensing pathways along ontogenesis in a porcine model.

Michael Oster, Eduard Murani, Cornelia C. Metges, Siriluck Ponsuksili and Klaus Wimmers

Plos One 2011, 6(7), e21691.

Conceived and designed the experiments: KW CCM SP. Performed the experiments: MO EM CCM SP KW. Analyzed the data: MO EM SP KW. Contributed reagents/materials/analysis tools: CCM KW SP EM. Wrote the paper: MO KW.

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A High Protein Diet during Pregnancy Affects Hepatic Gene Expression of Energy Sensing Pathways along Ontogenesis in a Porcine Model

Michael Oster[1], Eduard Murani[1], Cornelia C. Metges[2], Siriluck Ponsuksili[3], Klaus Wimmers[1]*

1 Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, **2** Research Unit Physiology of Nutrition, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, **3** Research Group Functional Genomics, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Abstract

In rodent models and in humans the impact of gestational diets on the offspring's phenotype was shown experimentally and epidemiologically. The underlying programming of fetal development was shown to be associated with an increased risk of degenerative diseases in adulthood, including the metabolic syndrome. There are clues that diet-dependent modifications of the metabolism during fetal life can persist until adulthood. This leads to the hypothesis that the offspring's transcriptomes show short-term and long-term changes depending on the maternal diet. To this end pregnant German landrace gilts were fed either a high protein diet (HP, 30% CP) or an adequate protein diet (AP, 12% CP) throughout pregnancy. Hepatic transcriptome profiles of the offspring were analyzed at prenatal (94 dpc) and postnatal stages (1, 28, 188 dpn). Depending on the gestational dietary exposure, mRNA expression levels of genes related to energy metabolism, N-metabolism, growth factor signaling pathways, lipid metabolism, nucleic acid metabolism and stress/immune response were affected either in a short-term or in a long-term manner. Gene expression profiles at fetal stage 94 dpc were almost unchanged between the diets. The gestational HP diet affected the hepatic expression profiles at prenatal and postnatal stages. The effects encompassed a modulation of the genome in terms of an altered responsiveness of energy and nutrient sensing pathways. Differential expression of genes related to energy production and nutrient utilization contribute to the maintenance of development and growth performance within physiological norms, however the modulation of these pathways may be accompanied by a predisposition for metabolic disturbances up to adult stages.

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* E-mail: wimmers@fbn-dummerstorf.de

Introduction

The relationship between the intrauterine adaptive response to adverse changes in the biological environment with persistent postnatal effects and permanent consequences on the phenotype is termed 'fetal programming'. Due to developmental plasticity, interactions between genotype and environment lead to adaptations in gene expression and ultimately to a modified phenotype [1]. In various human and animal studies it was shown that low maternal protein intake during pregnancy is associated to low birth weight, and subsequently to disorders of metabolic health as well as body composition of the offspring [2–5]. Interestingly, it has been demonstrated that a low protein diet fed to pregnant dams will lead to intrauterine growth retardation (IUGR) and reduced birth weight [6–10]. Moreover, there are cues that diet-dependent modifications of the metabolism induced during fetal life can persist until adulthood. These observations were summarized to generate the 'thrifty phenotype hypothesis' [11] suggesting that a poor fetal nutrient supply causes low birth weight and increases the risk for adult metabolic disease. Less is known about possible effects of gestational high protein diets on offspring development and later health. It has been previously shown in rats, mice, and pigs that maternal excess protein intake during pregnancy is associated to IUGR, and results suggest that this is related to net energy deficiency in the gestating dam [12–14]. Interestingly, epidemiological studies in women show that high protein intakes during pregnancy can also result in fetal growth retardation [15–17]. Here, we fed a very high protein excess (30% CP) in order to promote effects on the offspring at the molecular (transcriptome, proteome) and organismal level.

Altered circulating levels of a number of metabolically active hormones like insulin or cortisol and mitochondrial energy-producing pathways are involved in the short term regulatory and long-term adaptive processes association with nutritional programming [2,18–20]. Because the liver is the central metabolic organ and its activities are essential for storage, utilization, and partitioning of nutrients it is the major target of molecular mechanisms leading to the establishment of a metabolic memory. Studies on nutritional programming were mainly done in rodent animal models and most studies were focused on biochemical and transcriptional changes of selected candidate metabolic and/or signaling pathways and their related genes at particular developmental stages. However, longitudinal holistic studies of the modulation of the offspring transcriptome due to maternal dietary

supply during pregnancy are scarce. In order to contribute to a comprehensive inventory of genes and functional networks that are targets of nutritional programming initiated during fetal life, we applied whole-genome microarrays for expression profiling in a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages. A porcine model was used, where pregnant sows were fed either a gestational high protein diet or an adequate protein diet on an isoenergetic basis to investigate the effects on hepatic gene expression in their fetuses and offspring. We focussed on four key developmental stages to gain knowledge regarding the complex regulatory mechanisms between different dietary protein levels during pregnancy and the transcriptome level of the offspring.

There are multiple factors for the development of intrauterine growth retardation, including maternal gestational diets, which are an issue in pig breeding. In this context it is of interest that lower birth weight piglets (<1.2 kg) later on show lower daily gains, higher body fat contents and lower muscle mass [21]. Moreover, the study comprises a valuable model for nutritional programming in humans, since pigs and humans share similarity in metabolism, physiology, anatomy and genome [22,23]. These experimental data will complement existing findings from rodent models and epidemiological human data. In particular the high protein diet provides a model for dietary recommendations to combat obesity in human. The porcine model used here shows that the porcine offspring, which was exposed to an oversupply with protein during fetal development but had appropriate postnatal dietary conditions, was able to adapt in terms of their organismal phenotype [14,24,25]. In fact, in our experiment, newborns from sows that received a high protein supply during gestation had a significantly lower birth weight and in particular a lower body fat content than newborns of the control group. However, neither body weight, body composition, and cellularity of muscle and adipose tissue of weaning piglets, nor carcass weight, carcass composition, and meat quality at slaughter differed significantly among offspring of the HP and the AP group [14,24,25]. However, the transcriptomic analysis presented here, reveals that the hepatic expression profiles show altered responsiveness of energy sensing pathways and indicate a predisposition to metabolic disturbances up to adult stages.

Results

We performed a longitudinal holistic study of the hepatic transcriptome modulation due to maternal gestational dietary supply in order to provide a comprehensive inventory of genes and functional networks that are targets of fetal nutritional programming using a porcine model. Regarding the effect of 'fetal programming' pregnant sows were fed either a high protein diet (HP) containing 30% crude protein or an isocaloric adequate protein diet (AP) containing 12% crude protein. We investigated their offspring's hepatic gene expression in one prenatal and three postnatal stages with the help of porcine 24 k-microarrays. In total we found 12,477 probe-sets expressed at stage 94 dpc (1 dpn: 12,646; 28 dpn: 12,285; 188 dpn: 11,835) according to MAS5 analysis. Further filtering based on the variability of expression of probe-sets revealed 7,802 probe-sets for further analysis at stage 94 dpc (1 dpn: 10,253; 28 dpn: 7,971; 188 dpn: 8,950). These probes sets represent 5,887 genes at stage 94 dpc (1 dpn: 7,699; 28 dpn: 6,194; 188 dpn: 6,903) according to the recent annotation [26]. In order to identify molecular pathways affected by the gestational diets we first analysed differential expression between the dietary groups within each stage separately. The different dietary exposure of the offspring during prenatal development can be expected to cause slight shifts of the developmental age of the offspring that may be reflected by subtle changes of the transcriptome and could hamper the identification of direct effects of the gestational diets on the hepatic expression. Secondly, we analysed the differences among both experimental groups regarding the more long-term and more pronounced changes of expression patterns between the adjacent stages. In total we found 13,459 probe-sets expressed within 94 dpc and 1 dpn (1 dpn and 28 dpn: 13,557; 28 dpn and 188 dpn: 12,629) according to MAS5 analysis. After the filtering steps described above, 10,462 probe-sets were detected within 94 dpc and 1 dpn (1 dpn and 28 dpn: 10,636; 28 dpn and 188 dpn: 8,686). These probes sets represent 7,817 genes within 94 dpc and 1 dpn (1 dpn and 28 dpn: 7,940; 28 dpn and 188 dpn: 6,704). Notably, q-values between ontogenetic stages within diet were remarkable lower ($q \leq 0.05$) than between diets within stage ($q \leq 0.25$).

Comparisons between HP and AP within stages

Expression of mRNA was compared in HP and AP offspring within each ontogenetic stage (Figure 1). At stage 94 dpc 7 probe-sets differed significantly between HP and AP fetuses (1 increased). It was not possible to assign those genes to significant regulated metabolic pathways. In perinatal piglets (stage 1 dpn) 878 probe-sets differed between HP offspring (503 for HP>AP) and AP offspring. Ingenuity Pathway Analysis indicates enrichment of molecular routes related to energy metabolism, lipid metabolism, N-metabolism, cellular growth and immune response (Table 1). In particular, genes associated with oxidative phosphorylation (OXPHOS), biosynthesis of steroids, and valine, leucine and isoleucine degradation were found diminished in HP offspring. Furthermore, genes associated with RAN signaling as well as PPARGC1a and PRKAA2 showed an increased expression in HP offspring at stage 1 dpn. Analysis towards common regulation of genes higher expressed in HP than AP revealed 217 potential regulatory elements (RE) (177 for HP<AP) that corresponded to 103 transcription factors (TF) (107 for HP<AP). In juvenile piglets (stage 28 dpn) 498 probe-sets differed between HP and AP offspring. The expression of 274 probe-sets was increased in the HP offspring compared with AP offspring. At juvenile stage, pathways of lipid metabolism and N-metabolism were enriched. Genes associated with fatty acid metabolism, and valine, leucine and isoleucine degradation showed a decreased mRNA expression in HP offspring. Analysis using DiRE identified 125 RE within the genes up-regulated in HP (103 for HP<AP) that were associated with 101 TF (100 for HP<AP). In adult pigs (stage 188 dpn) 1,903 probe-sets were significantly different between HP and AP offspring. Of these 1,177 probe-sets showed higher expression and 726 probe-sets showed lower expression in HP than in AP. The mRNA expression levels of genes associated with glucocorticoid receptor signaling, RAN signaling, PPAR signaling and IGF-1 signaling as well as fatty acid elongation in mitochondria and mitochondrial dysfunction were increased in HP offspring. The genes up-regulated in HP compared to AP were related to 117 (118 for HP<AP) TF that fitted 448 potential RE (190 for HP<AP). No genes were found consistently differentially expressed between the groups along all examined stages. However, at 1 dpn and 188 dpn 142 probe-sets were differentially regulated in both stages between HP and AP.

Differences of longitudinal ontogenetic regulation among HP and AP offspring

Considering two adjacent ontogenetic stages within one treatment group, significantly regulated transcripts were determined. The resulting gene lists were compared between HP and

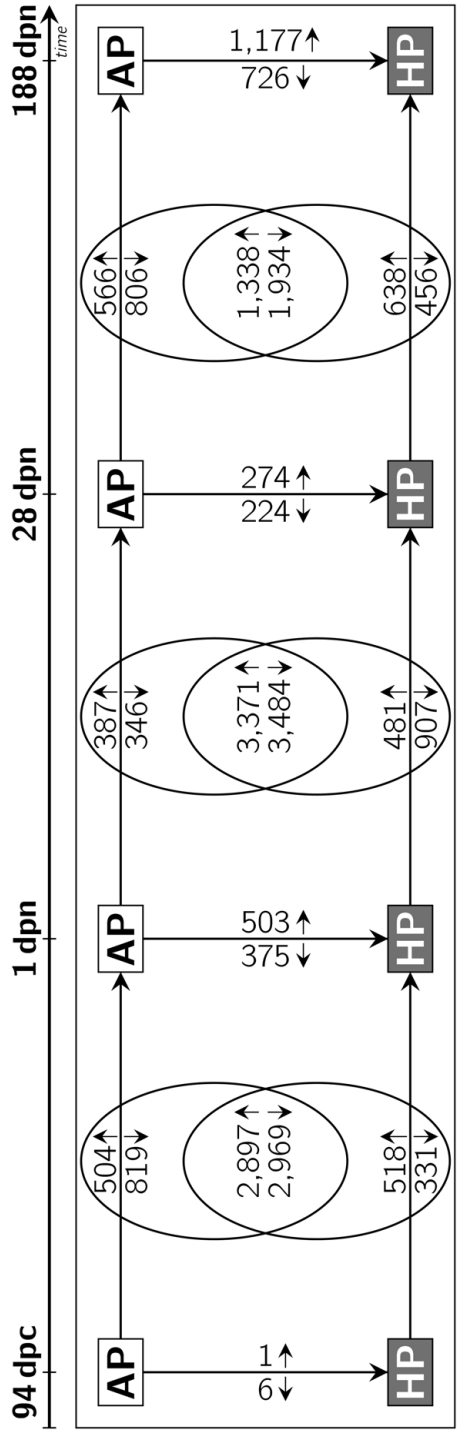

Figure 1. Number of regulated probe-sets in liver tissue. The numbers at the horizontal arrows indicate the quantity of probe-sets significantly regulated between the adjacent ontogenetic stages in either AP or HP offspring, whereas the numbers in the intersections indicate the quantity of probe-sets commonly regulated between stages in AP and HP offspring. The numbers at vertical arrows are the number of probe-sets differentially expressed between AP and HP offspring at the same ontogenetic stage (arrows between boxes show direction of the comparisons; small arrows to top = up-regulated, small arrows to bottom = down-regulated probe-sets).
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AP offspring at the corresponding ontogenetic periods. The intersection of commonly regulated genes between those comparisons was discarded because these regulations were likely due to physiologically developmental processes. Consequently only genes, whose regulation between two consecutive ontogenetic stages (period I: 94 dpc-1 dpn; period II: 1 dpn–28 dpn; period III: 28 dpn–188 dpn) was private to either the HP or the AP group were analysed. These genes display diet-dependent longitudinal transcriptomic regulation (Figure 1). Thus, genes and pathways identified as regulated in one offspring group were either unregulated or showed an opposite direction of regulation in the corresponding ontogenetic comparison within the other dietary group. Between fetal and perinatal stages (period I), there were 1,323 (504 for 1 dpn > 94 dpc in AP) probe-sets showing levels and directions of regulation in the AP group that were different from the HP group. The mTOR signaling was found to be increased at stage 1 dpn while genes associated with RAN signaling were decreased (Table 2). In HP offspring 849 probe-sets showed ontogenetic regulation (518 for 1 dpn > 94 dpc in HP) during the corresponding time period that is specific when compared to the AP group. The mRNA expression level of genes associated with glucocorticoid receptor signaling and RAN signaling in HP offspring was increased. Some genes participating in IGF-1 signaling, biosynthesis of steroids and growth hormone signaling were found to be decreased in HP perinatal piglets. DiRE pointed to 201 potential RE matching 106 TF for the genes that are up-regulated at 1 dpn compared to 94 dpc in AP (292 RE with 110 TF for 1 dpn < 94 dpc in AP; 217 RE with 97 TF for 1 dpn > 94 dpc in HP, 136 RE with 91 TF for 1 dpn < 94 dpc in HP). Comparing perinatal and juvenile piglets (period II) 733 probe-sets were regulated in a different manner in AP offspring than in HP offspring. Of these, 387 probe-sets showed up regulation and 346 probe-sets showed lower expression at higher age. Expression values of genes participating in AMPK signaling and the degradation of branched chain amino acids were increased, whereas genes associated with oxidative phosphorylation were decreased in AP offspring. In the same period 1,388 probe-sets exhibited ontogenetic regulation that was specific to HP offspring. Of these, 481 probe-sets showed an increased whereas 907 probe-sets showed a decreased mRNA expression. Genes involved in oxidative phosphorylation and growth hormone signaling were increased in HP offspring, while glucocorticoid receptor signaling was found to be down regulated. Those genes that were higher expressed at 28 dpn compared to 1 dpn in the AP group only exhibited 165 potential RE that were associated with 99 TF as revealed by in-silico analysis (175 RE with 107 TF for 28 dpn < 1 dpn in AP; 217 RE with 118 TF for 28 dpn > 1 dpn in HP, 305 RE with 93 TF for 28 dpn < 1 dpn in HP). When juvenile and young adult pigs (period III) are compared 1,372 probe-sets differed significantly (566 increased) in AP offspring. Genes higher expressed in older animals had 183 RE for 117 TF (254 RE with 103 TF for 188 dpn < 28 dpn in AP). Genes participating in glucocorticoid receptor signaling,

Table 1. Significantly regulated transcripts of metabolic pathways in liver tissue within different ontogenetic stages (Ingenuity Pathway Analysis).

Ontogenetic stage	Regulated pathway	Regulation	*P* value	No. of regulated genes	Genes involved in pathway
94 dpc	-	-	-	-	-
1 dpn	RAN signaling	up	4.57*E-7	6	IPO5, KPNA3, KPNB1, RAN, RANBP2, TNPO1
	Oxidative phosphorylation	down	2.01*E-15	23	ATP5H, ATP5I, ATP5L, ATP6V0D1, COX6A1, COX6B1, COX6B2, COX7A2, COX7B, COX8A, NDUFA3, NDUFA4, NDUFA13, NDUFB1, NDUFB5, NDUFB6, NDUFB7, NDUFB11, NDUFS7, NDUFS8, SDHB, TCIRG1, UQCR11
	Biosynthesis of steroids	down	1.19*E-10	10	DHCR7, EBP, FDFT1, FDPS, HMGCR, IDI1, MVK, NQO2, PMVK, SQLE
	Val, Leu, Ile degradation	down	3.41*E-2	4	ACAA1, ACAA2, ACAT2, HMGCS1
28 dpn	Val Leu, Ile degradation	down	6.50*E-4	5	ALDH1A1, ALDH7A1, HADHA, HMGCS1, IVD
	Fatty acid metabolism	down	9.55*E-3	5	ACOX1, ALDH1A1, ALDH7A1, HADHA, IVD
188 dpn	Glucocorticoid receptor signaling	up	1.75*10-3	25	A2M, AKT3, CD163, FOS, GRB2, GTF2A2, GTF2E2, GTF2F2, HSP90AA1, HSPA8, HSPA9, ICAM1, IL10, JAK1, MAP2K1, MAPK1, MAPK14, PPP3CB, RRAS2, STAT5B, SUMO1, TAF4, TAF7, TAF15, TGFBR2
	RAN signaling	up	8.21*10-3	4	IPO5, KPNA1, KPNA6, RAN
	IGF-1 signaling	up	3.62*10-3	12	AKT3, CSNK2A1, FOS, GRB2, IGF1, MAP2K1, MAPK1, PRKAR1A, RRAS2, YWHAG, YWHAQ, YWHAZ
	PPAR signaling	up	3.82*10-2	9	FOS, GRB2, HSP90AA1, IL18, MAP2K1, MAP4K4, MAPK1, RRAS2, STAT5B
	Fatty acid elongation in mitochondria	up	1.42*10-3	5	ACAA2, HADHB, HSD17B4, PECR, PPT1
	Mitochondrial dysfunction	up	3.63*10-3	12	AIFM1, APP, CYCS, GLRX2, MAOA, NDUFB3, NDUFB6, NDUFS1, PDHA1, PSEN1, SOD2, UQCRB

The comparison between the dietary gestational protein levels (HP vs. AP) is shown in dependence of the regulatory direction (up or down).
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RAN signaling, mTOR signaling and PPAR signaling were found to be down regulated. In HP offspring 1,094 probe-sets were differently expressed (638 for 188 dpn > 28 dpn in HP). In HP offspring genes involved in signaling pathways of IGF-1, AMPK, growth hormone and mTOR were up regulated as well as genes associated with fatty acid metabolism and degradation of branched chain amino acids. These genes covered 219 RE associated with 99 TF (160 RE with 105 TF for 188 dpn < 28 dpn in HP). Furthermore, genes involved in purine and pyrimidine metabolism were found to be down regulated. Figure 2 gives a comprehensive overview about the pathways found regulated between stages and diets.

Among TFs found to be associated to the genes regulated due to maternal gestational diets at various developmental stages of the offspring 40 were redundantly found among the top 10 TFs highlighted by DiRE for the various comparisons made. Only HNF4A, DR1, STAT6, and TCF4 themselves were found differentially regulated due to diet and stage.

For all genes exemplarily analysed qRT-PCR confirmed the direction of differential regulation as obtained by microarray analysis. In 80% of the genes that we validated by qRT-PCR, significant expression differences in mRNA levels between the treatment groups were identified by both qRT-PCR and microarray analysis. Correlations between expression values of microarray and qRT-PCR ranged between 0.43 and 0.84 and were highly significant (Table 3). This suggests that our microarray data are reliable.

Discussion

We applied whole-genome microarrays to evaluate offspring hepatic expression profiling affected by exposure to maternal isocaloric pregnancy diets with adequate or high protein concentrations. In order to investigate transcriptional features of developmental nutritional programming we conducted a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages in a porcine model. The comparisons of the relative mRNA abundances depending on dietary group and ontogenetic stages provide an overall view of the developmental plasticity of the liver. In the overall experimental design sampling of the heaviest and lightest offspring at 94 dpc and 1 dpn was foreseen. At these early stages the offspring depended on supply provided by the mother subjected to the experimental treatment. At later stages, 28 dpn and 188 dpn, when non-maternal, environmental effects become more relevant the animals close to the middle of the distribution for growth were used. At all stages sampling was balanced for sex. Impact of IUGR and low birth weight as well as sex on postnatal growth is well documented [27,28], however, body weight and sex affected the expression of only a few probe-sets (Table S1). Following in utero exposure to a gestational high protein diet we could not find significant regulatory changes of metabolic pathways in porcine fetal liver; however, at the whole-body level growth restricted HP offspring was observed [14]. At this stage the fetuses are subjected to the intrauterine environment for already a long time and on the level of the transcriptome HP offspring has adapted, whereas the

Table 2. Significantly regulated transcripts of metabolic pathways in liver tissue between two ontogenetic stages within one dietary group (Ingenuity Pathway Analysis).

Ontogenetic comparison	Diet	Regulated pathway	Regulation	No. of regulated genes	Genes involved in pathway	*P* value
94 dpc vs. 1 dpn	AP	mTOR signaling	up	3.42*E-3	9	EIF3I, GNB1L, INSR, MRAS, PRKAA1, PRKCB, RHOG, RHOU, VEGFB
	AP	RAN signaling	down	1.43*E-2	3	KPNA6 RANBP2 TNPO1
	HP	Glucocorticoid receptor signaling	up	4.44*E-3	13	A2M, ERCC3, ESR1, GTF2H3, HMGB1, HSPA1B, MAPK9, PIK3C2A, TAF2, PIK3R1, PIK3R3, PRKACB, PTGES3
	HP	RAN signaling	up	4.93*E-3	3	CSE1L, KPNA3, TNPO1
	HP	IGF-1 signaling	down	2.13*E-3	6	FOS, IGFBP3, JUN, PIK3C2B, YWHAB, YWHAE
	HP	Biosynthesis of steroids	down	1.02*E-2	3	DHCR7, MVK, PMVK
	HP	Growth hormone signaling	down	1.33*E-2	4	FOS, IGFBP3, PIK3C2B, SOCS4
1 dpn vs. 28 dpn	AP	AMPK Signaling	up	1.89*E-4	9	AK1, AK7, AKT3, HMGCR, IRS1, MAPK14, PRKAA2, PRKAB2, PRKAG2
	AP	Val, Leu, Ile degradation	up	3.87*E-3	5	ABAT, ACAT1, BCKDHA, HMGCS1, IVD
	AP	Oxidative phosphorylation	down	2.19*E-3	8	ATP6V0E2, ATP7B, COX17, COX10, NDUFA1, NDUFB6, NDUFS8, TCIRG1
	HP	Oxidative phosphorylation	up	3.70*E-4	11	ATP6V1D, ATP6V1F, ATP6V1H, NDUFA2, NDUFA7, NDUFB1, NDUFB7, NDUFB11, NDUFS3, NDUFS7, TCIRG1
	HP	Growth hormone signaling	up	1.24*E-2	5	FOS, IGFBP3, PIK3CG, PTPN6, STAT1
	HP	Glucocorticoid receptor signaling	down	3.12*E-2	15	CDKN1A, JAK2, KRAS, MAP3K7, MNAT1, NCOA1, NR3C1, PCK2, SMAD2, SOS2, TAF5, TAT, TBP, VCAM1
28 dpn vs. 188 dpn	AP	Glucocorticoid receptor signaling	down	1.01*E-2	17	BCL2, GTF2E2, HMGB1, HSPA2, HSPA9, HSPA14, MAP3K1, MAPK14, TBP, NCOA1, NCOR1, PIK3R1, PPP3CA, PRKACB, RAC3, STAT5B, SUMO1
	AP	mTOR signaling	down	2.97*E-2	10	EIF4E, EIF4G2, EIF4G3, PIK3R1, PPP2CA, PPP2CB, PPP2R1B, PRKCB, RAC3, RHOU
	AP	RAN Signaling	down	1.82*E-2	3	CSE1L, RAN, XPO1
	AP	PPAR signaling	down	3.69*E-2	7	CITED2, MAP4K4, NCOA1, NCOR1, PDGFRA, PPARA, STAT5B
	HP	IGF-1 signaling	up	2.56*E-5	11	AKT3, CTGF, IGF1, IGF1R, IGFBP3, IRS1, PRKAG2, PRKCZ, PTPN11, RRAS2, SOS2
	HP	mTOR signaling	up	2.73*E-6	15	AKT3, DDIT4, EIF3E, EIF4A2, EIF4B, FNBP1, IRS1, PPP2R5A, PRKAB2, PRKAG2, PRKCZ, RND3, RRAS2, STK11, VEGFA
	HP	AMPK signaling	up	6.87*E-3	9	AKT3, IRS1, PPM1A, PPM1D, PPP2R5A, PRKAB2, PRKAG2, SRC, STK11
	HP	Fatty acid metabolism	up	9,37*E-3	9	ACAA1, ACADL, ACADSB, ACAT1, AUH, CYP2C18, CYP51A1, IVD, PTGR1
	HP	Val, Leu, Ile degradation	up	5.89*E-5	9	ACAA1, ACADL, ACADSB, ACAT1, AUH, DBT, HIBCH, IVD, PCCA
	HP	Growth hormone signaling	up	6.03*E-3	6	IGF1, IGF1R, IGFBP3, IRS1, PRKCZ, SOCS6
	HP	Purine metabolism	down	1.90*E-4	17	ABCC1, AK2, APRT, ATP13A2, BAT1, CANT1, MYO9B, NME1, NSF, PKM2, PNPT1, POLR1E, POLR2G, POLR3G, PRPS2, RRM1, RUVBL2
	HP	Pyrimidine metabolism	down	1.31*E-2	8	CANT1, DKC1, NME1, PNPT1, POLR1E, POLR2G, POLR3G, RRM1

The comparison between dietary gestational protein levels (AP, HP) is shown in dependence of the regulatory direction (up or down).
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transcriptome of HP offspring might be significantly different at earlier fetal stages, i.e. transcriptome alterations may have preceded phenotype alterations. In perinatal piglets growth retardation was obvious among all HP offspring [14] - in the subset of animals used for the microarray analysis HP offspring showed numerically lower mean birth weight, however the differences among the HP and AP offspring failed to be significant. Anyhow, at 1 dpn the mRNA expression levels were extensively altered. At this time point the piglets are subjected to major nutritional changes that require acute adaptive regulation of the transcriptome. The adaptability to modulate expression profiles during porcine fetal development in response to a given

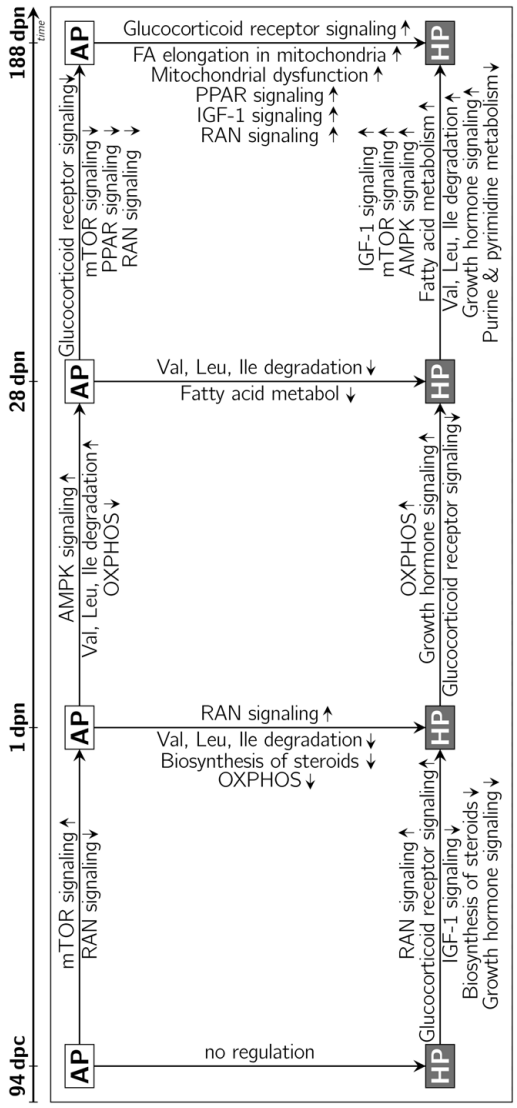

Figure 2. Regulated pathways in liver tissue between ontogenetic stages and maternal diets. Listed pathways between stages in AP offspring (white boxes) indicate the appropriate ontogenetic development, which doesn't occur in HP offspring (black boxes) at the corresponding developmental period. Pathways between the HP stages indicate processes and metabolic regulations, which occur in the HP offspring but not in the AP offspring in the corresponding developmental period. The differences in gene regulation dependent on diet and ontogenetic stage indicate fetal programming in terms of developmental and metabolic disorders (arrows between boxes show direction of the comparisons; small arrows to top = up-regulated, small arrows to bottom = down-regulated pathways; mTOR, mammalian target of rapamycin; RAN, Ras-related nuclear protein; IGF-1, insulin-like growth factor; PPAR, peroxisome proliferator receptor; AMPK, AMP-activated kinase).
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environment seems to be quite high. This flexibility might depend on the degree of maturation and it might contribute to unchanged transcriptome levels at fetal stage. Alterations of the hepatic transcriptome profile in perinatal HP offspring may indicate stress and assign a programmed respond on new environmental cues.

The data provide a snapshot of the transcriptome, thus it remains unclear to what extent transcriptomic differences observed at pre- and perinatal stages between the experimental groups are due to chronic long-term effects or to acute stimulating event in life, like birth or weaning. Interestingly, offspring at 28 dpn showed a reduced number of differentially expressed mRNAs compared to stage 1 dpn. Also at this time point HP offspring may has adapted its expression profiles to the early postnatal feeding conditions during weaning time. However, the number of differentially expressed probe-sets increased again at adult stage. Our model indicates that gestational HP diets affected the expression profiles in a short-term as well in a long-term manner in HP offspring. The effect is characterized by an engagement of the genome that leads to different responsiveness and adaptability of the gene expression machinery to chronic and acute environmental stimuli. Due to the HP diet a number of energy sensing, producing and utilising pathways are important in these processes.

The impact of the maternal diets was obvious at the transcriptomic level in the respective offspring, though not effects on traits related to the development of the whole organism or single organs were observed at 1 dpn (in the subset of animals used), 28 and 188 dpn. This indicates that there is a direct relationship between the dietary treatments of the sows and the expression profiles of the offspring. Obviously, the modulations of the expression profiles are not consequences of impaired growth due to the experimental diets. Epigenetic effects manifesting during crucial pre- and perinatal developmental stages are regarded as mechanisms promoting nutritional (environmental) impact on the genome leading to shifted gene expression [29,30]. Especially DNA-methylation is a mechanism of epigenetic gene regulation that might be involved [31]. Our study supports the identification of genes that are targets of epigenetic mechanisms of gene regulation. The analysis of RE and TF common to DE-genes only revealed four TF that were themselves regulated due to the dietary treatments. Together with the finding that DE-genes were mainly functionally annotated to cellular signaling pathways and metabolic pathways, this may be interpreted as an indication that the transcriptional response to prenatal nutritional challenge is primarily present at the level of these cellular signaling and metabolic routes rather than at hierarchically superior genes encoding transcription factors of regulatory networks acting at the DNA- and RNA-level.

Oxidative phosphorylation and mitochondrial metabolic pathways

For developmental and metabolic processes an effective energy supply is required. Generation of ATP via oxidative phosphorylation is the most efficient way for energy supply [32,33]. Mitochondria account for 20% of the liver cell volume and are exquisitely sensitive to environmental changes regarding efficiency and accuracy of mitochondrial replication [34]. The observed regulation of the oxidative phosphorylation (OXPHOS) at stage 1 dpn (Figure 2) may be considered as stress response due to a new life situation. The nutrient supply of the neonatal piglets changes from the parenteral route via the umbilical cord and some swallowing of amniotic fluid to enteral nutrition with colostrum containing compounds new to the individual (e.g. fat, lactose, immunoglobulins) [35]. This may cumulate in an altered

Table 3. Comparison of microarray data and qRT-PCR of selected transcripts.

Gene name	Microarray			qRT-PCR#			Correlation##
	p-value	FC	Regulation	p-value	FC	Regulation	Expr. values
1 dpn							
PPARGC1A	0.0103	+1.54	up	0.012	+1.63	up	0.51***
PRKAA2	0.0354	+1.15	up	0.007	+1.47	up	0.43**
HMGCR	0.0177	−1.53	down	0.030	−2.01	down	0.84***
DHCR7	0.0004	−1.57	down	0.001	−1.76	down	0.63***
NDUFA4	0.0081	−1.19	down	0.310	−1.15	n.r.	0.75***
NDUFS7	0.0097	−1.20	down	0.480	−1.14	n.r.	0.51***
NDUFS8	0.0223	−1.22	down	0.030	−1.32	down	0.56***
188 dpn							
PPARGC1A	0.9341	+1.01	n.r.	0.275	+1.22	n.r.	0.77***
PRKAA1	0.0083	−1.35	down	0.055	−1.38	down	0.74***
GADD45B	0.0766	+1.49	up	0.047	+1.81	up	0.67***

#calculated by factorial normalization on RPL10 expression values;
##p-value of Spearman's rho; n.r. - not regulated.
**$p<0.01$;
***$p<0.001$.
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metabolic status in terms of OXPHOS. The down regulation of genes associated to the mitochondrial respiratory chain suggests a possibly disturbed electron transfer within the OXPHOS. Disturbed mitochondrial oxidative phosphorylation could be of relevance regarding the growth retardation observed for neonates of the HP group compared to the AP group [36]. This might be accompanied by an altered mitochondrial production of reactive oxygen species. The mitochondrial alterations are possibly to be accompanied by a decreased energy production resulting in an altered AMP/ATP-ratio. Transcript levels of the cellular energy sensor AMPK (AMP-dependent protein kinase) showed an increased expression (PRKAA2). AMPK is targeting various genes contributing to growth related signal cascades as well as fuel dependent pathways like fatty acid oxidation or steroid synthesis [37,38]. The increased expression of PRKAA2 in HP offspring at stage 1 dpn could be part of a compensation process due to the reduced expression of genes involved in the oxidative phosphorylation and a possibly reduced cellular ATP content. Consistently, key enzymes of the energy consuming steroid biosynthesis, HMGCR and DHCR7, showed a decreased expression at stage 1 dpn in HP offspring. A decreased synthesis of important cellular compounds like cholesterol may occur. The transcription factor PGC-1α (Peroxisome proliferator-activated receptor γ coactivator-1α) is another downstream, but not directly regulated effector protein of AMPK, which regulates multiple aspects of cellular energy metabolism [39,40]. Although PGC-1α is known to act as a positive regulator on the mitochondrial biogenesis and the expression of ROS-detoxifying enzymes [41], the diet-dependent mRNA expression of PPARGC1A led neither to an adaptation of processes related to mitochondrial dysfunction nor an increased transcription of antioxidative enzymes. This may also indicate the snapshot character of the microarray analysis, because the increased expression of PPARGC1A contributes to a compensatory process, when levels of PRKAA2 as well as OXPHOS associated genes showed an equal mRNA expression at stage 28 dpn. The diet-dependent diametrical alteration of OXPHOS associated genes leads to the conclusion that the ability of porcine offspring to balance metabolic modulation is remarkably high. Because the integrity of mitochondria is seen as an important factor for metabolic health [42–44], diet-dependent alterations affecting transcriptional expression patterns of mitochondria related pathways are of interest. Therefore, the susceptibility of OXPHOS to be down regulated as a response to environmental factors (stage 1 dpn) as well as the up regulation of genes associated with mitochondrial dysfunction in HP offspring (stage 188 dpn) may contribute to the development of a predisposition to degenerative diseases in terms of fetal programming after a maternal protein excess. Moreover, at stage 188 dpn in HP offspring a major activator of mitochondrial metabolism, PRKAA1 encoding AMPK, showed diminished expression. This may also contribute to the predisposition to metabolic diseases because AMPK is targeted by antidiabetics [37,45]. Moreover, the increased expression of genes associated with fatty acid elongation in mitochondria in HP offspring at stage 188 dpn may contribute to mitochondrial alterations. The fatty acid elongation in mitochondria takes place in the mitochondrial matrix and is essentially the reverse of the beta-oxidation by acting primarily on fatty acyl-CoA substrates shorter than 16 carbons. Furthermore, there were clues for a diet-dependent transcriptional alteration of genes associated with the lipid metabolism in HP offspring, including fatty acid metabolism and PPAR signaling. These modulations might contribute to compensatory regulations in terms of growth and body composition. Taken together, these findings suggest that energy sensing pathways and their signals are important in the adaptive response to prenatal nutritional environment.

Signaling of intracellular metabolism

The mTOR signaling acts as an important nutrient sensing pathway that controls protein synthesis in mammalian cells at the level of translation [46]. Upstream signaling events of mTOR signaling include alterations in amino acid availability, abundance of hormones, AMP and growth factors [47]. Thus, mTOR signaling is involved in regulating individual cell growth, growth

performance, and developmental processes [48,49]. An increase of mTOR signaling from fetal to neonatal stage was observed in AP offspring with no change in the HP group. This may account for a reduced protein synthesis in HP offspring and thus, contribute to the observed growth retardation and altered metabolic status as proposed by Inoki et al. [50]. In addition, mTOR signaling might be also involved in compensatory growth processes. Therefore, the diet-dependent diametrical regulation of mTOR signaling associated genes between juvenile and adult pigs can be interpreted as successful compensation in terms of growth performance. The success of compensatory processes in HP offspring becomes obvious by the fact that their weight was lowered at stages 94 dpc and 1 dpn compared to the AP group, but similar at stage 188 dpn.

It is well documented that IGF-1 signaling pathway plays an essential role in fetal survival and mediates postnatal growth as well as developmental and ageing processes [51–53]. Most of the IGF-1 mediated anabolic pleiotropic effects are mediated through association with IGF-1 receptor [54]. With respect to mRNA expression of hepatic IGF-1 signaling the impact of this pathway in terms of growth retardation and possibly compensatory regulations can be estimated. Regarding gestational low protein diets many studies showed an altered IGF-1 signaling [5,55,56]. Therefore, it is of interest that mRNA expression of IGF-1 signaling associated genes was regulated due to gestational HP diets, too. When adjacent ontogenetic stages of both experimental groups were compared, only HP offspring showed altered expression profiles regarding IGF-1 signaling. The down regulation of IGF-1 signaling between fetuses and perinatal piglets in HP offspring may underlay the observed low birth weight in HP offspring. Furthermore, the increased mRNA expression of genes associated with IGF-1 signaling after weaning can be considered as compensatory effects in terms of growth and body weight regulation. Interestingly, the up regulation of IGF-1 signaling took place in a time period, when mRNA expression levels of OXPHOS associated genes, and thus probably the energy production, were within a normal range at stage 28 dpn in HP offspring. Since there is an association between the level of growth factor and the sensitivity of cells to this growth factor, the increased expression of IGF-1 signaling at stage 188 dpn may go along with an increase in insulin sensitivity at adult stage. Notably, other studies investigating effects of gestational dietary protein level showed a high insulin responsiveness in early postnatal stages but a lowered one during adulthood [7,57,58].

The growth hormone signaling, another anabolic growth factor signaling, regulates metabolic processes including protein synthesis and has important regulatory effects on protein, carbohydrate and lipid metabolism [59]. The mRNA levels of growth hormone signaling related transcripts showed biphasic regulation during the development of HP offspring. Therefore, the observed down regulation of growth hormone signaling associated genes at birth and their up regulation towards juvenile and adult stages correspond with the growth retardation at stages 94 dpc and 1 dpn and the compensatory growth onwards in adulthood.

Glucocorticoids, a major subclass of steroid hormones, regulate a large number of metabolic, behavioural, cardiovascular and immune functions. Their biologic effect is modulated by the glucocorticoid receptor with succeeding direct and indirect interactions of downstream target genes to modulate gene expression. The glucocorticoid receptor signaling terminates stress reactions and mobilizes energy resources for this purpose. The mRNA expression of genes associated with glucocorticoid receptor signaling was shown to be dependent on maternal gestational protein diets [2,60,61]. The biphasic regulation of glucocorticoid receptor signaling in HP prenatal, perinatal and juvenile offspring underlines the postulated increased stress response in HP offspring at birth, which may cumulate in the observed disturbances of mitochondrial activity. Furthermore, the diet-dependent regulation of glucocorticoid receptor signaling between juvenile and adult stage and the resulting increased glucocorticoid receptor signaling at stage 188 dpn in HP offspring suggests that HP offspring was in a kind of alarm alert (activated defence) at adult stage.

Cell maintenance and proliferation

Cell maintenance and proliferation RAN, a member of the Ras family of small GTPases, is a positive key regulator of mitosis [62] and plays a critical role in multiple cellular functions, including nucleocytoplasmic transport, nuclear envelope assembly and spindle formation during the cell cycle [63]. Therefore, the RAN signaling is involved in cellular growth and developmental processes. The diet and stage dependent expression of RAN signaling was partly diametrically regulated along the ontogenetic development (between stages 94 dpc vs. 1 dpn and within stage 1 dpn), which represents adaptation to different prenatal conditions. An almost similar direction of regulation of genes involved in RAN signaling was found at juvenile and adult stages (between stages 28 dpn vs. 188 dpn and within stage 188 dpn), which may account for compensatory processes.

The pathways concerning the metabolisms of purine and pyrimidine are related to a number of processes including nucleotide biosynthesis, degradation and salvage. The down regulation of genes associated with the metabolisms of purine and pyrimidine probably account for a reduced cellular turnover at stage 188 dpn compared to stage 28 dpn in HP offspring. GADD45B, a gene whose transcription is induced in response to multiple environmental and physiological stress factors, is involved in DNA repair, apoptosis, cell survival and growth arrest [64]. The mRNA expression of GADD45B was increased in HP offspring at stage 188 dpn, which suggests a metabolic priority for cell survival and a terminated compensatory growth performance at adult stage.

Valine, leucine and isoleucine are indispensable branched-chain amino acids. The catabolism of all three amino acids shares the same enzymes during the first steps, including transamination and decarboxylation. As final result, three different CoA derivates are produced, which can be directed towards the synthesis of steroids or ketone bodies and to the citrate cycle. The down regulation of genes associated with the valine, leucine and isoleucine degradation in HP offspring at stages 1 dpn and 28 dpn may correspond to a higher demand for these amino acids than in AP offspring. However, HP offspring increased the expression of genes involved in the degradation of valine, leucine and isoleucine during post weaning development which resulted in an unaltered expression level at stage 188 dpn between the dietary groups.

Conclusion

Gestational HP diets affected the hepatic expression profiles at prenatal and postnatal stages. The effects encompass a bias of the genome leading to altered responsiveness of energy and nutrient sensing pathways. Obviously the programming of the genomes warrants the adaptations and compensatory growth, but probably at the expense of the predisposition for metabolic disturbances up to adult stage. In order to test this hypothesis the porcine model could be used in an experiment where offspring of sows fed different gestational diets is also objected to different dietary challenges at postnatal stages.

Materials and Methods

Animals and sample collection

Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection and the experimental protocol was approved by the Animal Care Committee of the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg - Vorpommern, Germany; LVL MV/TSD/7221.3-1.1-006/04; LALLF M-V/TSD/7221.3-1.2-05/06; LALLF M-V/TSD/7221.3-1.2-013/06). The animal experiment was performed as described [14]. A high protein diet (HP) containing 30% (w/w) crude protein or an adequate protein diet (AP) containing 12% crude protein were formulated to be isocaloric (13.6 MJ ME/kg on average). The gilts of both groups consumed 2.6 kg/d resulting in a significantly different protein intake but equal energy intake [14]. The gestation diets did not affect reproductive parameters like litter size and litter weight as well as percentage of stillborn and mummies [14]. At insemination German Landrace primiparous sows (n = 48) were randomly assigned to either the HP or the AP group (Figure 3). Tissue sampling included offspring of these sows at one prenatal (94 dies post conceptionem (dpc)) and three postnatal time points (1, 28, 188 dies post natum (dpn)). At d 94 of gestation, a subset of eight sows per dietary group was subjected to Caesarean section (EXP1). Sows were anesthetized as described [14]. This experiment was performed over 5 replicates. Eight viable foetuses per sow were collected starting at the tip of the left uterus horn and alternating between left and right horn. Fetuses were killed by i.v. injection of T61 (Intervet, Unterschleissheim, Germany) in the V. cava cranialis and liver samples were immediately collected (approximately 500 mg), frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until analysis. Fetuses originated from litters of at least 11 viable piglets. Fetuses of HP fed dams showed a decreased weight compared to AP fetuses at 94 dpc (HP: 622 ± 119 g, and AP: 711 ± 118 g, respectively; $P \leq 0.05$; n = 32). The smallest and the heaviest fetus were selected for transcriptome analysis. In the second experiment (EXP2) offspring selected for the postnatal time points was born to primiparous sows after prostaglandin induction of parturition as described [14] and farrowed after a mean pregnancy duration of 115 days. This experiment was conducted over 8 replicates and offspring of a subset of 4 sows (2 per diet per replicate) with a minimum of 11 live born piglets (median litter size = 13) was used. At birth 10 piglets in each litter were distributed over three time points (1, 28, 188 dpn). For the microarray analyses, 8 sib pairs, which were balanced for sex (all stages) and discordant for weight (light and heavy piglet; stages 94 dpc and 1 dpn only) were chosen per stage and diet. Thirty-six hours after birth, the lightest and the heaviest piglet within one litter were killed by i.m. injection of 1.25 mg propionyl-promazine (0.2 ml Combelen, Bayer AG, Leverkusen, Germany) and 50 mg ketamine (Ursotamin, Serumwerk Bernburg AG, Germany). Samples were immediately collected from lobus sinister hepaticus (approximately 500 mg), frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until analysis. The remaining piglets were cross-fostered to non-experimental sows of 2nd to 4th parities, which were on the AP diet during gestation. All sows were fed AP lactation diets. Litter size during suckling was standardized to 11 piglets per sow. Male piglets were castrated at d 4 of age. From weaning (28 dpn) to slaughter (188 dpn), all piglets were individually reared. They had free access to standard diets formulated for post-weaning (d 29 to d 76), growing (d 77 to d 105) and finishing periods [65] and had the same feed intake. At 28 dpn and 188 dpn of age, pigs were weighed after an overnight fast and killed by electronarcosis followed by exsanguination in the experimental slaughterhouse of FBN. Liver tissue was immediately collected from lobus sinister hepaticus, frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until use for RNA isolation. In our experiment piglets (1 dpn) born from HP sows had significantly lower birth weight (HP: 1.21 ± 0.04 kg, and AP: 1.41 ± 0.04 kg respectively, $P \leq 0.05$) that mainly resulted from reduced body fat, whereas body mass index and ponderal index did not differ from the AP group [14]. Mean birth weights of animals of the HP group used for microarray analyses were numerically but not significantly lower than birth weights of AP offspring (HP: 1.31 ± 0.31 kg, and AP: 1.36 ± 0.31 kg, respectively; $P = 0.31$; n = 32). At 28 dpn, the animals of the AP and HP group showed no difference in body weight (HP: 7.36 ± 1.54 kg, and AP: 7.59 ± 2.14 kg respectively; $P = 0.37$; n = 32) and body composition, i.e. muscle, fat depots, bones, skin, as well as in analytical components (protein, fat, ash, moisture) or structural and biochemical properties of fat and muscle tissues [25]. Also at 188 dpn offspring of AP and HP sows did not differ significantly in body weight (HP: 131.41 ± 7.11 kg, and AP: 131.55 ± 15.11 kg respectively; $P = 0.31$; n = 32). Like at earlier ages animals of the HP group tended have lower meat but higher fat percentage, but the differences were not significant [24].

RNA isolation, target preparation and hybridization

According to the manufacturer's protocol total RNA from individual liver samples was isolated using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany). After DNase treatment a column-based purification using the RNeasy Mini Kit (Qiagen,

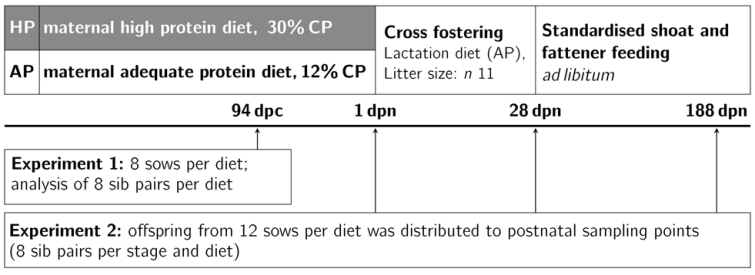

Figure 3. Experimental design.
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Hilden, Germany) was done. The RNA samples were visualized on 1% agarose gels containing ethidium bromide to check RNA integrity. RNA was quantified by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). To ensure the absence of a DNA contamination within the isolated RNA a PCR amplification with the porcine glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was done (Forward primer: AAGCAGGGATGATGTTCTGG; Reverse primer: ATGCCTCCTGTACCACCAAC). All RNA samples were stored at $-80^{\circ}C$ until downstream analysis was performed. For the microarray experiments individual biotin-labeled cRNA was synthesized by the Gene Chip 3 Express Kit (Affymetrix, Santa Clara, CA, USA). The cRNA was fragmented ($\approx 100bp$) and hybridized on Affymetrix GeneChip porcine Genome Array. After staining and washing steps the arrays were scanned (Affymetrix, Santa Clara, CA, USA).

Data analysis

The programming was done in R using Bioconductor [66]. Firstly, a quality control was performed. At 94 dpc 15 AP-samples met the appropriate quality control criteria (94 dpc-HP: 16; 1 dpn-AP: 15; 1 dpn-HP: 16; 28 dpn-AP: 14; 28 dpn-HP: 15; 188 dpn-AP: 16; 188 dpn-HP: 16 samples). Samples were GCRMA normalized (Log2). The MAS5 algorithm was used to skip those transcripts which were expressed in less than 50% of the animals within one dietary group per stage. For a second filtering step standard deviations were calculated for each probe-set over all subsets of arrays of the particular comparisons. Probe-sets with a low standard deviation ($s \leq 0.25$) were discarded, because such transcripts are not likely to be regulated. Relative changes in mRNA levels were determined using a mixed model analysis, including effects of dietary treatment, sex, mother, weight (for stages 94 dpc and 1 dpn) and interaction between sex and dietary treatment ($v_{ijkl} = diet_i + sex_j + mother_k(diet_i) + weight_l + (diet\,x\,sex)_{ij} + error_{ijkl}$). P-values (significance set at $P \leq 0.05$) for each stage were converted to a set of q-values ($q \leq 0.25$) using the algorithm proposed by Storey and Tibshirani [67]. In general, results are given for the comparisons in the direction of HP vs. AP; thus 'up regulation' or 'increased expression' indicates higher expression in HP than in AP. Analysis of the pathways involved was carried out using Ingenuity Pathway Analysis [68]. The up-to-date annotation of Affymetrix probe-sets to EnsEMBL Sscofa 9 was used. The Affymetrix GeneChip Porcine Genome Array contains 24,123 probe-sets that interrogate 20,689 transcripts that were assigned to known genes [26]. All the microarray data is MIAME compliant and the raw data has been deposited in a MIAME compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) (accession numbers: GSE25482 and GSE25483).

Pathway analysis

Gene lists from microarray results were submitted to the manually curated database 'Ingenuity Pathways Analysis' to elucidate putative pathways associated with an altered gene expression in porcine liver. The focus was on those canonical pathways which came up at least once within the top ten regulated pathways within one single analysis. It should be noted here that the interactions presented in the networks are not specific for porcine liver tissue, as the database contains literature from many different research areas. The web server named DiRE (for predicting distant regulatory elements; access at http://dire.dcode.org; [69]) was used to determine common transcription factor binding sites (TFBS) of the diet-dependent regulated genes.

Quantitative real-time RT-PCR

First-strand cDNA was synthesized from $2\mu g$ of total RNA (n = 14 per diet and stage) using random primers and oligo d(T) 13VN in the presence of Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). In order to survey expression of the liver tissue samples, total transcript levels of selected target and reference genes (Table 4) were quantified by real-time quantitative PCR (qPCR) performed on a LightCycler 480 system using the LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany). The amplification was conducted in duplicate according to manufacturer's instructions using $10\mu M$ of each primer. Reactions were performed in a final volume of $10\mu l$ using $5.0\mu l$ of LightCycler 480 SYBR Green I Master (Roche), $2.0\mu l$ of Aqua dest., $10\mu M$ ($0.5\mu l$) of each primer (Table 4) and $40ng$ ($2\mu l$) cDNA. The temperature profiles comprised an initial denaturation step at $95^{\circ}C$ for 10 min and 40 cycles consisting of denaturation at $95^{\circ}C$ for 15 s, annealing at $60^{\circ}C$ for 10 s and extension/

Table 4. Primer used to verify microarray experiments in liver tissue by qRT-PCR.

Gene name	Probe set ID	Sequence 5′ – 3′	Accession no.	T(°C)	Size (bp)
DHCR7	Ssc.5455.1.S1_at	For GCATGACACTGACTTCTTCTC Rev CCCACCTCCACTTTATTC	BE232966	60	136
GADD45B	Ssc.14764.1.A1_at	For GGACTTAGACTTTGGGACTTG Rev GTAAGCCTCCCATCTCTCTT	BF708594	60	140
HMGCR	Ssc.16088.1.S1_at	For GTGCTGGTCTGTTTTGATTT Rev TGCAGTGATTTGTTTTCTTG	BP436947	60	159
NDUFA 4	Ssc.7315.1.S1_at	For TCCTGCTTAGTCCCCGACCTT Rev ACAGTGCTGCTCCAGTACCTCC	CF793329	60	164
NDUFS 7	Ssc.1681.1.S1_at	For CGGCTACTACCACTACTCCT Rev ATCCGCAGTCTCTTCTCC	CK455535	60	156
NDUFS 8	Ssc.2312.1.S1_at	For AGTTTGTGAACATGCGTGAG Rev CTCAAATGGGTAGTTGATGG	BI181006	60	152
PPARGC1A	Ssc.16864.1.S1_at	For GTAAATCTGCGGGATGATGG Rev TGGTGGAAGCAGGATCAAAG	AB106108	60	208
PRKAA 1	Ssc.8107.1.A1_at	For TTGTTAATTTCATAAACTTTGCTTC Rev GTGCAGCCTTGACATACTC	BF712533	60	193
PRKAA 2	Ssc.16257.1.S1_at	For TCTGTAATTCTGTTTTGCCTACG Rev AGCAAGAAGGTGATGCCAAG	NM214266	60	168
RPL 10*	Ssc.9130.1.A1_at	For CTGTGTTCGTCTTTTCTTCC Rev TCATCCACTTTTGCCTTCT	BI181297	60	199

DHCR7 - 7-dehydrocholesterol reductase; GADD45B - growth arrest and DNA-damage-inducible, beta; HMGCR - 3-hydroxy-3-methylglutaryl-coenzyme A reductase; NDUFA4 - NADH-ubiquinone oxidoreductase MLRQ subunit; NDUFS7 - NADH-ubiquinone oxidoreductase 20 kDa subunit; NDUFS8 - NADH-ubiquinone oxidoreductase 23 kDa subunit; PPARGC1A - Peroxisome proliferator activated receptor gamma coactivator 1 alpha; PRKAA1 – 5′-AMP-activated protein kinase, catalytic alpha-1 chain; PRKAA2 – 5′-AMP-activated protein kinase, catalytic alpha-2 chain; RPL10 - Ribosomal protein 10.
*House keeping gene.
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fluorescence acquisition at $72^{\circ}C$ for 15 s. For all the assays threshold cycles were converted to copy numbers using a standard curve generated by amplifying serial dilutions of an external PCR standard (10^7-10^2 copies). 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 effciency 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, 28 individual liver mRNA samples were analyzed in duplicate per stage. Data were analyzed using the PROC MIXED, including effects of treatment, sex, mother, birth weight and interaction between sex and treatment (SAS version 9.1; SAS Institute, Cary, NC). Differences were considered significant at $P \leq 0.05$.

Supporting Information

Table S1 Significantly regulated probe-sets due sex at various stages.
(XLS)

Author Contributions

Conceived and designed the experiments: KW CCM SP. Performed the experiments: MO EM CCM SP KW. Analyzed the data: MO EM SP KW. Contributed reagents/materials/analysis tools: CCM KW SP EM. Wrote the paper: MO KW.

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2.3 A low protein diet during pregnancy provokes a lasting shift of hepatic expression of genes related to cell cycle along ontogenesis in a porcine model.

Michael Oster, Eduard Murani, Cornelia C. Metges, Siriluck Ponsuksili and Klaus Wimmers

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A low protein diet during pregnancy provokes a lasting shift of hepatic expression of genes related to cell cycle throughout ontogenesis in a porcine model

Michael Oster[1], Eduard Murani[1], Cornelia C Metges[2], Siriluck Ponsuksili[3] and Klaus Wimmers[1*]

Abstract

Background: In rodent models and in humans the impact of gestational diets on the offspring's phenotype was shown experimentally and epidemiologically. Adverse environmental conditions during fetal development provoke an intrauterine adaptive response termed 'fetal programming', which may lead to both persistently biased responsiveness to extrinsic factors and permanent consequences for the organismal phenotype. This leads to the hypothesis that the offspring's transcriptome exhibits short-term and long-term changes, depending on the maternal diet. In order to contribute to a comprehensive inventory of genes and functional networks that are targets of nutritional programming initiated during fetal life, we applied whole-genome microarrays for expression profiling in a longitudinal experimental design covering prenatal, perinatal, juvenile, and adult ontogenetic stages in a porcine model. Pregnant sows were fed either a gestational low protein diet (LP, 6% CP) or an adequate protein diet (AP, 12% CP). All offspring was nursed by foster sows receiving standard diets. After weaning, all offspring was fed standard diets *ad libitum*.

Results: Analyses of the hepatic gene expression of the offspring at prenatal (94 *dies post conceptionem*, dpc) and postnatal stages (1, 28, 188 *dies post natum*, dpn) included comparisons between dietary groups within stages as well as comparisons between ontogenetic stages within diets to separate diet-specific transcriptional changes and maturation processes. We observed differential expression of genes related to lipid metabolism (e.g. Fatty acid metabolism, Biosynthesis of steroids, Synthesis and degradation of ketone bodies, FA elongation in mitochondria, Bile acid synthesis) and cell cycle regulation (e.g. Mitotic roles of PLK, G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation). Notably, at stage 1 dpn no regulation of a distinct pathway was found in LP offspring.

Conclusions: The transcriptomic modulations point to persistent functional demand on the liver towards cell proliferation in the LP group but not in the AP group at identical nutritional conditions during postnatal life due to divergent 'programming' of the genome. Together with the observation that the offspring of both groups did not differ in body weight but in body composition and fat content, the data indicate that the activity of various genes led to diverse partitioning of nutrients among peripheral and visceral organs and tissues.

Background

Pregnancy and fetal development are periods of rapid growth and cell differentiation when mother and offspring are vulnerable to changes in dietary supply. Adverse environmental conditions during fetal development provoke an intrauterine adaptive response termed 'fetal programming', which may lead to both persistently biased responsiveness to extrinsic factors and permanent consequences for the organismal phenotype [1-6]. Due to developmental plasticity, environmental factors induce altered expression of the genome and ultimately modify the offspring's phenotype [7]. In various human and animal studies a gestational low protein intake during pregnancy was accompanied by low birth weight offspring, which was subsequently predisposed for metabolic disorders and alterations in body composition

* Correspondence: wimmers@fbn-dummerstorf.de
[1]Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany
Full list of author information is available at the end of the article

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[6,8-10]. Interestingly, epidemiological studies in women showed that maternal malnutrition during pregnancy can result in fetal growth retardation [11,12].

In order to contribute to a comprehensive inventory of genes and functional networks that are targets of nutritional programming initiated during fetal life, we applied whole-genome microarrays for expression profiling in a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages in a porcine model. On an isoenergetic basis, pregnant sows were fed either a gestational low protein diet (LP, 6% CP) or an adequate protein diet (AP, 12% CP) to investigate the effects on hepatic gene expression in their fetuses and offspring. The experiment comprises a valuable model especially for 'fetal' programming, because cross-fostering enabled assessment of solely the nutritional effects during gestation. Moreover, due to the similarity in metabolism, physiology, anatomy and genome the study is also a beneficial model for nutritional programming in humans [13,14]. Thus the experimental data will complement previous findings from rodent models and epidemiological human data. In particular, the low protein diet provides a model for prenatal dietary undersupply and exposure to famine that regrettably still burdens a considerable proportion of the human population.

In our experiment the porcine offspring, which was exposed to an undersupply of protein during fetal development but had appropriate postnatal dietary conditions, was able to broadly adapt in terms of their body weight. In fact, newborns from sows that received a low protein supply during gestation had a significantly lower birth weight, a lower body fat content, reduced size and number of adipocytes and muscle fibres than newborns of the control group. At weaning (28 dpn) offspring of the LP group showed slight but significant higher fat content and adipocyte size, and still lower muscle fibre numbers. But neither body weight at weaning nor body weight at 188 dpn differed significantly among offspring of the LP and the AP group, whereas visceral and subcutaneous fat content remained higher in LP than in AP during postnatal life [15-17]. Here the focus is on the hepatic transcriptomic response. We present that the hepatic expression profiles showed considerable modulation during prenatal and postnatal stages, i.e. in acute and delayed response to the nutritional stimulus. Nutritional fetal programming becomes apparent as an altered hepatic expression of genes related to cell cycle and cell maintenance, and lipid, ketone body, and amino acid metabolism, indicating different functional demands and replies of the liver in both experimental groups under identical nutritional conditions *post natum*.

Results

We performed a longitudinal holistic study of the hepatic transcriptome of offspring of dams fed either an experimental low protein diet (LP) or an adequate protein diet (AP) throughout gestation, in order to obtain a comprehensive picture of genes and functional networks that are sensitive to fetal nutritional programming using a porcine model. We investigated the offspring's hepatic gene expression at 94 dpc, 1 dpn, 28 dpn and 188 dpn by 24 k-microarray analysis. In total we found 12,650 probe-sets expressed at stage 94 dpc (1 dpn: 12,005; 28 dpn: 12,307; 188 dpn: 11,784) according to MAS5 analysis. Further filtering based on the variability of expression of probe-sets revealed 7,937 probe-sets for further analysis at stage 94 dpc (1 dpn: 9,099; 28 dpn: 8,250; 188 dpn: 8,943). These probe-sets represent 5,887 genes at stage 94 dpc (1 dpn: 6,965; 28 dpn: 6,387; 188 dpn: 6,958), according to the recent annotation [18]. In order to identify molecular pathways affected by the gestational diets we first analysed differential expression between the dietary groups within each stage separately. The different dietary exposure of the offspring during prenatal development can be expected to cause slight shifts of the developmental age of the offspring that may be reflected by subtle changes of the transcriptome and could hamper the identification of direct effects of the gestational diets on the hepatic expression. Secondly, we analysed the differences among both experimental groups regarding the more long-term and more pronounced changes of expression patterns between the adjacent stages. In total we found 13,357 probe-sets expressed within 94 dpc and 1 dpn (1 dpn and 28 dpn: 13,259; 28 dpn and 188 dpn: 12,637) according to MAS5 analysis (Table 1). After the filtering steps described above, 10,293 probe-sets were detected within 94 dpc and 1 dpn (1 dpn and 28 dpn: 10,317; 28 dpn and 188 dpn: 8,892). These probe-sets represent 7,697 genes within 94 dpc and 1 dpn (1 dpn and 28 dpn: 7,758; 28 dpn and 188 dpn: 6,879). Notably, q-values between ontogenetic stages within diet were remarkable lower ($q \leq 0.05$) than between diets within stage ($q \leq 0.25$).

Comparisons between LP and AP within stages

Expression of mRNA was compared in LP and AP offspring within each ontogenetic stage (Figure 1). At stage 94 dpc 1,001 probe-sets differed significantly between LP and AP fetuses (541 LP ≻ AP). Ingenuity Pathway Analysis indicates enrichment of molecular routes related to genetic information and nucleic acid processing and cell cycle that were found to be diminished, whereas the 'Wnt signaling' was found to be increased in LP offspring (Table 2). In perinatal piglets (stage 1

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Table 1 No. of expressed probe-sets, filtered probe-sets, and regulated probe-sets of LP and AP offspring at distinct developmental stages and periods

No. of expressed probe-sets	No. of filtered probe-sets	No. of total regulated probe-sets	No. of regulated probe-sets private to LP offspring	No. of regulated probe-sets private to AP offspring	No. of commonly regulated probe-sets of LP and AP offspring (intersection)
94 dpc					
12,650	7,937	1,001 (541 up, 460 down)			
1 dpn					
12,005	9,099	1 (0 up, 1 down)			
28 dpn					
12,307	8,250	483 (214 up, 269 down)			
188 dpn					
11,784	8,943	2,084 (952 up, 1,132 down)			
developmental period 1					
13,357	10,293	8,166 (3,731 up, 4,435 down)	1,042 (384 up, 658 down)	1,034 (503 up, 531 down)	6,090 (2,844 up, 3,246 down)
developmental period 2					
13,359	10,317	8,329 (4,118 up, 4,211 down)	881 (423 up, 458 down)	991 (448 up, 543 down)	6,457 (3,247 up, 3,210 down)
developmental period 3					
12,637	8,892	6,612 (2,810 up, 3,803 down)	1,959 (834 up, 1,125 down)	1,549 (697 up, 852 down)	3,104 (1,279 up, 1,825 down)

Developmental period 1 indicates the comparisons between stages 94 dpc and 1 dpn; Developmental period 2 indicates the comparisons between stages 1 dpc and 28 dpn; Developmental period 3 indicates the comparisons between stages 28 dpc and 188 dpn; 'Up' indicates higher expression values of later stages, 'down' indicates lower expression values at later stages.

dpn) 1 probe-set differed between LP offspring and AP offspring (0 increased). Therefore, no significant regulated metabolic pathway was determined.

In juvenile piglets (stage 28 dpn) 483 probe-sets differed between LP and AP offspring. The expression of 214 probe-sets was increased in the LP offspring compared with AP offspring. Genes associated with the 'complement system' showed increased mRNA expression levels, whereas the canonical pathway 'G1/S checkpoint regulation' was found to be decreased in LP offspring.

At adult age (stage 188 dpn) 2,084 probe-sets were significantly different between LP and AP offspring. Of these, 952 probe-sets showed higher expression and 1,132 probe-sets showed lower expression in LP than in AP offspring. The mRNA expression levels of genes associated with metabolic processing of ketones, fatty acids, bile acids, and hydrophobic amino acids (Val, Ile, Leu) as well as 'mTOR signaling', 'VEGF signaling', and 'glucocorticoid receptor signaling', were increased in LP offspring at stage 188 dpn, whereas 'biosynthesis of steroids' was found to be diminished.

No genes were found consistently differentially expressed between the groups along all examined stages. However, at 94 dpc and 188 dpn 179 probe-sets were differentially regulated in both stages between LP and AP.

Differences of longitudinal ontogenetic regulation among LP and AP offspring

Considering two adjacent ontogenetic stages within one treatment group, significantly regulated transcripts were determined. The resulting gene lists were compared between LP and AP offspring at the corresponding ontogenetic periods. The intersection of commonly regulated genes between those comparisons was discarded because regulation of these genes was likely due to physiologically developmental processes. Consequently, only genes whose regulation between two consecutive ontogenetic stages (period I: 94dpc-1dpn; period II: 1dpn-28dpn; period III: 28dpn-188dpn) was private to either the LP or the AP group were analysed (Table 1). These genes display diet-dependent longitudinal transcriptomic regulation (Figure 1). Thus, genes and pathways identified as regulated in one offspring group were either unregulated or showed an opposite direction of regulation in the corresponding ontogenetic period within the other dietary group.

Between fetal and perinatal stages (period I), there were 1,034 (503 1 dpn ≻ 94 dpc) probe-sets showing

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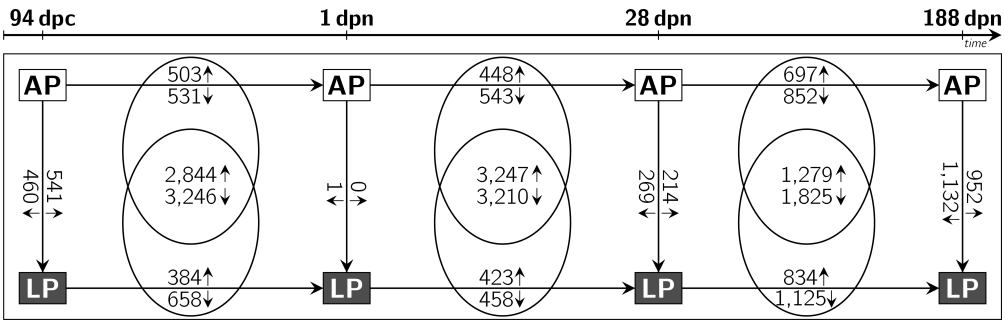

Figure 1 Number of regulated probe-sets in liver tissue. The numbers at the horizontal arrows indicate the quantity of probe-sets significantly regulated between the adjacent ontogenetic stages in either AP or LP offspring, whereas the numbers in the intersections indicate the quantity of probe-sets commonly regulated between stages in AP and LP offspring. The numbers at vertical arrows are the number of probe-sets differentially expressed between AP and LP offspring at the same ontogenetic stage. (Arrows between boxes show direction of the comparison; small arrows indicate up and down regulation, respectively).

Table 2 Significantly regulated transcripts of metabolic pathways in liver tissue within different ontogenetic stages (Ingenuity Pathway Analysis).

Ontogenetic stage	Regulated pathway	Direction of regulation	*P* value	No. of regulated genes	Genes involved in pathway
94 dpc	Wnt signaling	up	1.20*E-2	9	ACVR1, CSNK1G3, FZD4, FZD6, MAP3K7, MMP7, TCF4, TCF7L2, WNT5A
	Mitotic roles of Polo-like kinase	down	5.15*E-8	11	CCNB1, CCNB2, CDC23, CDC25B, CDK1, FZR1, SP90AA1, KIF11, PLK1, PPP2R1B, PTTG1
	G1/S checkpoint regulation	down	2.76*E-5	8	CCND2, CCND3, CCNE1, CDK4, E2F1, E2F4, RB1, TFDP1
	G2/M DNA damage checkpoint regulation	down	1.53*E-3	5	CCNB1, CCNB2, CDC25B, CDK1, PLK1
1 dpn	-	-	-	-	-
28 dpn	Complement system	up	2.07*E-5	5	C4B, C5, C6, C9, CD55
	G1/S checkpoint regulation	down	2.49*E-2	3	CDKN1B, HDAC11, TGFB3
188 dpn	VEGF signaling	up	2.80*E-4	12	ACTA2, AKT3, BCL2, EIF2B1, KDR, MAPK1, MRAS, PIK3R3, PRKCB, RRAS2, VCL, VEGFC
	mTOR signaling	up	6.29*E-3	13	AKT3, EIF3B, EIF4B, MAPK1, MRAS, PIK3R3, PPP2CB, PRKAB2, PRKCB, RHOJ, RRAS2, TSC1, VEGFC
	Synthesis and degradation of ketone bodies	up	1.18*E-3	4	ACAA1, BDH1, HADHA, HADHB
	Bile acid synthesis	up	3.33*E-3	7	ACAA1, ADH5, ADHFE1, ALDH7A1, HADHA, HADHB, LIPA
	Fatty acid elongation in mitochondria	up	4.81*E-3	4	ACAA1, AUH, HADHA, HADHB
	Glucocorticoid receptor signaling	up	3.99*E-2	17	AKT3, BCL2, CCL2, GTF2A2, GTF2B, GTF2E2, HSP90AB1, HSPA1B, IL1RN, MAPK1, MRAS, NCOR1, PBX1, PIK3R3, RRAS2, TAF4, VCAM1
	Val, Leu, Ile degradation	up	8.32*E-3	8	ACAA1, ACAD10, ALDH7A1, AUH, BCKDHB, HADHA, HADHB, HIBADH
	Biosynthesis of steroids	down	7.65*E-3	5	CYP24A1, CYP7B1, DHCR7, FDFT1, MVD

The comparison between the dietary gestational protein diets (LP vs. AP) is shown in dependence of the regulatory direction (up or down).

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levels and directions of regulation in the AP group that were different from the LP group. Genes associated with 'AMPK signaling' were found to be increased at stage 1 dpn, while expression of genes associated with 'G2/M DNA damage checkpoint regulation', 'mitotic roles of Polo-like kinase' and 'pyrimidine metabolism' were decreased (Table 3). In LP offspring 1,042 probe-sets showed ontogenetic regulation (384 1 dpn ≻ 94 dpc) during the corresponding time period that was group-specific. The mRNA expression level of genes associated with cell cycle, mitosis, and metabolism of purines and pyrimidines was increased in LP offspring. Furthermore, genes participating in 'Wnt signaling' were found to be decreased in LP perinatal piglets.

Comparing perinatal and juvenile piglets (period II) 991 probe-sets were regulated in a different manner in AP offspring and in LP offspring. Of these, 448 probe-sets were up-regulated and 543 probe-sets showed lower expression at a higher age. Expression values of genes participating in 'AMPK signaling' and 'mTOR signaling' were increased, whereas genes associated with the 'degradation of valine, leucine and isoleucine' were decreased in AP offspring. In the same period 881 probe-sets exhibited ontogenetic regulation that was specific to LP offspring. Of these, 423 probe-sets showed an increased mRNA expression. Genes involved in 'fatty acid metabolism', 'biosynthesis of steroids', 'synthesis and degradation of ketone bodies', 'glucocorticoid receptor signaling' as well as the 'degradation of valine, leucine and isoleucine' were found to be up-regulated in LP offspring, while genes associated with 'G1/S checkpoint regulation' were down-regulated.

When juvenile and young adult pigs (period III) are compared, 1,549 probe-sets differed significantly (697 188 dpn ≻ 28 dpn) in AP offspring. Genes participating in 'AMPK signaling' and 'fatty acid metabolism' were found to be up-regulated while genes associated with 'mitotic roles of Polo-like kinase' and 'VEGF signaling' were found to be down-regulated in AP offspring. In LP offspring 1,959 probe-sets were differently expressed (834 188 dpn ≻ 28 dpn). In LP offspring, genes involved in 'mTOR signaling', 'fatty acid elongation in mitochondria' as well as the 'degradation of valine, leucine and isoleucine' were up regulated. Furthermore, genes involved in 'actin cytoskeleton signaling', 'RhoA signaling', 'Rac signaling' and 'complement system' were found to be down-regulated. Figure 2 gives a comprehensive overview of the pathways found regulated between stages and diets. For all genes exemplarily analysed, qRT-PCR confirmed the direction of differential regulation as obtained by microarray analysis. In 90% of the genes that we validated by qRT-PCR, significant expression differences in mRNA levels between the treatment groups were identified by both qRT-PCR and microarray analysis (Table 4). Correlations between expression values of microarray and qRT-PCR of positive validated transcripts ranged between 0.47 and 0.86 and were highly significant. This suggests that our microarray data are reliable.

Discussion

We applied whole-genome microarrays to evaluate hepatic gene expression profiles of offspring from sows fed either an isocaloric maternal low protein or adequate protein diet throughout their pregnancy. In order to investigate transcriptional features of developmental nutritional programming we conducted a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages in a porcine model. The comparisons of the relative mRNA abundances depending on dietary group and ontogenetic stages provide an overall view of the developmental plasticity of the liver.

At the prenatal stage mRNA expression profiles were extensively altered between the dietary groups. At this time point the fetuses were subjected to a serious nutritional deficiency that requires an acute transcriptomic response. Following in utero exposure to a gestational low protein diet at 1 dpn we could not find significant regulatory changes of genes of any molecular route in porcine perinatal liver. At this stage the animals experienced a kind of release from metabolic burden after cross-fostering and suckling. Obviously, this initiates an immediate recovery of the activity of genes to 'normal level' reflected by similar expression pattern in LP and AP piglets. However, at the whole-body level adverse consequences of restricted intrauterine supply were observed [15]. In juvenile LP offspring, mRNA expression profiles were moderately altered compared to AP offspring. However, the number of differentially expressed probe-sets increased extensively at adult stage. The observed transcriptional postnatal regulations were delayed long-term effects of the prenatal nutritional supply. Due to the normal dietary conditions at juvenile and adult stages the alterations found here can be regarded as fetal nutritional programming.

Our model indicates that gestational LP diets affected the hepatic expression profiles in an acute, short-term as well as in a delayed, long-term manner in LP offspring. Due to the LP diet a number of molecular routes related to cell cycle and cellular turnover, response to stimuli, as well as energy-, lipid- and amino acid metabolic pathways are shifted on the transcriptional level in the liver at prenatal stages under the direct influence of limited protein and/or amino acid supply, but also postnatal. The effect is characterised by a programming of the genome that leads to different responsiveness and adaptability of the gene expression machinery to chronic and acute environmental stimuli, i.e. nutritional supply.

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Table 3 Significantly regulated transcripts of metabolic pathways in liver tissue between two ontogenetic stages within one dietary group (Ingenuity Pathway Analysis)

Ontogenetic comparison	Diet	Regulated pathway	Direction of regulation	*P* value	No. of regulated genes	Genes involved in pathway
94 dpc vs. 1 dpn	**AP**	AMPK signaling	up	6.96*E-3	8	AKT2, INSR, PIK3C2A, PIK3R1, PPM1B, PRKAA1, PRKACB, RAC1
	AP	Mitotic roles of Polo-like kinase	down	3.25*E-4	7	CCNB1, CCNB2, CDC25B, CHEK2, KIF11, PLK4, PTTG1
	AP	Pyrimidine metabolism	down	2.52*E-3	10	CAD, DCTD, DKC1, POLQ, POLR3E, POLR3K, RFC5, RRM1, TYMS, UCK2
	AP	G2/M DNA damage checkpoint regulation	down	2.04*E-4	6	CCNB1, CCNB2, CDC25B, CHEK2, UBC, YWHAE
	LP	Purine metabolism	up	9.20*E-3	12	DDX39, PNPT1, POLA2, POLE2, POLR1B, POLR1C, POLR2I, RFC3, RRM2, RRM2B, RUVBL1, RUVBL2
	LP	Pyrimidine metabolism	up	6.15*E-7	14	CTPS, PNPT1, POLA2, POLE2, POLR1B, POLR1C, POLR2I, PUS1, RFC3, RRM2, RRM2B, TXNRD1, TYMS, UCK2
	LP	Mitotic roles of Polo-like kinase	up	7.55*E-5	7	ANAPC4, CDC27, CDK1, ESPL1, FZR1, PPP2R1B, PTTG1
	LP	G2/M DNA damage checkpoint regulation	up	3.09*E-2	3	CDK1, CHEK1, YWHAZ
	LP	Wnt signaling	down	4.02*E-2	9	AKT3, FZD5, MMP7, NLK, SOX4, TCF3, TCF4, TCF7L2, WNT5A
1 dpn vs. 28 dpn	**AP**	AMPK signaling	up	4.26*E-3	8	AK1, CPT1A, EIF4EBP1, HMGCR, MAPK14, NOS3, PRKAA2, PRKAB2
	AP	mTOR signaling	up	1.89*E-2	7	EIF3F, EIF3G, EIF4EBP1, FNBP1, GNB1L, PRKAA2, PRKAB2
	AP	Val, Leu, Ile degradation	down	6.40*E-3	6	ACAD8, ACADL, ACADSB, BCAT1, DBT, MCCC2
	LP	Val, Leu, Ile degradation	up	7.12*E-8	11	ACADSB, ACAT1, ACAT2, ALDH1A1, AUH, BCKDHB, GCDH, HMGCL, HMGCS1, MCCC2, MCEE
	LP	Fatty acid metabolism	up	3.23*E-3	8	ACADSB, ACAT1, ACAT2, ALDH1A1, AUH, CYP51A1, GCDH, PECI
	LP	Synthesis and degradation of ketone bodies	up	1.76*E-6	5	ACAT1, ACAT2, BDH2, HMGCL, HMGCS1
	LP	Biosynthesis of steroids	up	3.27*E-4	5	CYP24A1, FDPS, HMGCR, IDI1, SC5DL
	LP	Glucocorticoid receptor signaling	up	3.57*E-3	12	CDKN1C, CXCL3, IL10, MAP3K1, NCOA2, NFKBIB, NR3C1, POLR2B, PRKACB, RRAS2, SLPI, SMARCA4
	LP	G1/S checkpoint regulation	down	2.22*E-2	4	CCNE1, CDC25A, CDKN1A, E2F3
28 dpn vs. 188 dpn	**AP**	AMPK signaling	up	1.50*E-3	10	INSR, MAPK12, PPAT, PPM1A, PPP2CA, PPP2R3A, PRKAA2, SMARCA2, SRC, STK11
	AP	Fatty acid metabolism	up	1.28*E-2	8	ACADSB, ALDH1A1, CYP1B1, CYP2D6, CYP3A4, CYP4A11, CYP4B1, PECI
	AP	Mitotic roles of Polo-like kinase	down	8.33*E-3	7	ANAPC5, CDK1, PLK1, PLK2, PPP2R1B, SLK, WEE1
	AP	VEGF signaling	down	7.3*E-3	9	AKT3, BCL2, KDR, NOS3, PRKCB, RAC2, RRAS, VCL, VEGFC
	LP	Val, Leu, Ile degradation	up	1.07*E-2	7	ACAA1, ACAT1, ALDH1B1, AUH, ECH1, IVD, IWS1
	LP	Fatty acid elongation in mitochondria	up	2.05*E-2	3	ACAA1, AUH, ECH1
	LP	mTOR signaling	up	2.65*E-2	10	AKT3, DDIT4, EIF4B, EIF4G3, PPP2R1B, PRKAB2, PRKAG1, PRKAG2, TSC1, VEGFA
	LP	Actin cytoskeleton signaling	down	7.99*E-5	24	ACTB, ACTR2, ARPC4, ARPC1A, CD14, CFL1, F2R, FGD1, LBP, MAP2K1, MYH9, NCKAP1, PAK2, PIK3C3, PIK3C2A, PIK3R4, PIKFYVE, PPP1R12A, RDX, ROCK1, ROCK2, TMSB4X, TMSL3, WASF1
	LP	RhoA signaling	dow	5.05*E-3n	12	ACTB, ACTR2, ARHGAP1, ARPC4, ARPC1A, CFL1, PIKFYVE, PPP1R12A, RDX, ROCK1, ROCK2, WASF1

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Table 3 Significantly regulated transcripts of metabolic pathways in liver tissue between two ontogenetic stages within one dietary group (Ingenuity Pathway Analysis) *(Continued)*

LP	Rac signaling	down	4.34*E-13	ACTR2, ARPC4, ARPC1A, CFL1, MAP2K1, NCKAP1, PAK2, PIK3C3, PIK3C2A, PIK3R4, PIKFYVE, WASF1
LP	Complement system	down	2.17*E-2	C2, C7, C9, CFB, MBL2

The comparison between the dietary gestational protein diets (LP vs. AP) is shown in dependence of the regulatory direction (up or down).

Together with the observation that the offspring of both groups did not differ in bodyweight but in body composition and fat content [17], the data indicate that the activity of different genes led to different partitioning of nutrients among peripheral and visceral organs and tissues.

Transcriptional excursions regarding cell maintenance and proliferation

Mammalian cell division is precisely regulated by variate factors and functional networks. Therefore, cell division is synchronous with cell growth [19,20]. Eukaryotic cells evolved elaborate mechanisms to verify the fidelity of cell division. Therefore, a cell cycle control system can arrest the cycle at certain checkpoints. Key components in terms of cell cycle regulation are cyclins. Cyclin levels undergo an oscillation of synthesis and degradation in each cell cycle and fall at a proper developmental time point to exit the cell cycle [21,22]. Therefore, the transcriptional control of cyclins provides an additional level of growth regulation. Mitotic cyclins interact with Polo-like kinases (PLK), an evolutionary conserved family of essential cell cycle regulators, which are required at several key points within mitosis, including entry into and exit from mitosis [23,24]. It has been shown that fetal growth retardation is accompanied by alterations in cell cycle regulating molecules at both transcriptome [25] and proteome level [26]. However, longitudinal studies focussed on diet-dependent alterations of cell cycle regulators are scarce. The porcine expression patterns in this study showed a decreased mRNA expression of genes associated with the control of mitosis and cell cycle checkpoint regulation at stage 94 dpc in LP fetuses. These regulations may indicate an impaired fetal growth performance due to the gestational low protein diet. Therefore, the down-regulation of cell cycle

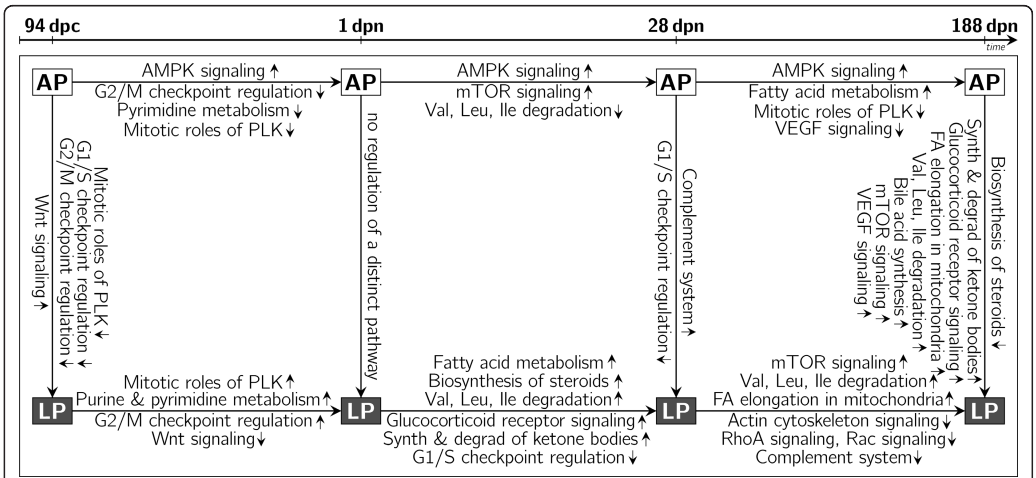

Figure 2 Regulated pathways in liver tissue between ontogenetic stages and diets. Listed pathways between AP stages (white boxes) indicate the appropriate ontogenetic development, which does not take place in the LP offspring (black boxes) at the corresponding developmental period. Pathways between the LP stages indicate processes and metabolic regulation, which occur in the LP offspring but not in the AP offspring in the corresponding developmental period. The differences in gene regulation dependent on diet and ontogenetic stage indicate fetal programming in terms of developmental and metabolic disorders (arrows between boxes show direction of the comparison; small arrows indicate up and down regulation, respectively; PLK, Polo-like kinase; VEGF, Vascular endothelial growth factor, mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; RhoA, Ras homolog gene family, member A; Rac, Ras-related C3 botulinum toxin substrate; FA, Fatty acid)

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regulating pathways might lead to a lengthened cell cycle in LP fetuses and offspring [27] and cumulate in growth retardation in LP fetuses. In AP fetuses, cell cycle parameters were found to be decreased in expression within developmental period I, which indicates a terminated fetal growth. In contrast, LP fetuses showed an increased expression of transcripts associated with cell division within the corresponding developmental period. Obviously, the increased mRNA expression of genes related to cell cycle regulators within developmental period I in LP offspring accounts for compensatory regulations regarding the lowered fetal weight due to the gestational diet. Within developmental period III, AP offspring showed a decreased expression of genes associated with Polo-like kinases, which is in line with the assumption that LP pigs have not terminated their growth at stage 188 dpn; in particular their liver has not attained a steady-state and that there is persistent functional demand towards cell proliferation potentially to respond to an elevated metabolic burden, including a higher turn over of lipids and ketone bodies.

The expression level of *NCAPG* (non-structural maintenance of chromosomes (SMC) condensin I complex subunit G) was highlighted as strongly associated with fetal growth retardation [28,29]. According to recent studies *NCAPG* is important during mitotic cell division. Due to the gestational low protein diet *NCAPG* was found to be down-regulated in LP fetuses compared to AP fetuses, which reflects the organismal effort to counteract the growth retarding processes in LP fetuses.

Beside pathways related to the control of growth and cell deviation, the diet-dependent expression patterns showed regulation in genes associated with biosynthesis, degradation and salvage of nucleotides ('purine and pyrimidine metabolism') as well as cell motility and cytokinesis ('actin cytoskeleton signaling', 'Rac signaling', 'RhoA signaling'). These expression patterns suggest a dietary effect on cellular turn-over, which might be increased at early stages but decreased at adult stage in LP offspring. Therefore, a diet-dependent tissue remodelling might take place in LP offspring as observed previously [30].

The 'mTOR signaling' acts as an important nutrient sensing pathway that controls protein synthesis in mammalian cells at the level of translation [31]. Upstream signaling events of 'mTOR signaling' include alterations in amino acid availability, abundance of hormones, AMP and growth factors [32]. Thus, 'mTOR signaling' is involved in regulating individual cell growth, growth performance, and developmental processes [33,34]. An increase of 'mTOR signaling' within developmental period II was observed in AP offspring, but not in the LP group. This may account for improper protein synthesis [35] in LP offspring. However, at adult stage a diet-dependent up-regulation of 'mTOR signaling' in LP offspring suggests a transcriptional priority for cellular growth and proliferation. Therefore, 'mTOR signaling' might be involved in compensatory growth processes at adult stage in LP offspring.

Another signaling pathway identified to be regulated in a diet-dependent manner is the 'VEGF (vascular endothelial growth factor) signaling' pathway, which is known from gene deletion studies to be essential in developmental processes [36,37]. Therefore, the transcriptional up-regulation of 'VEGF signaling' reflects the effort on cell maintenance and angiogenic growth in LP offspring at stage 188 dpn. Obviously, hepatic

Table 4 Comparison of microarray data and qRT-PCR of selected transcripts

Gene name	Microarray			qRT-PCR #			Correlation ##
	p-value	FC	Regulation	p-value	FC	Regulation	Expr. values
94 dpc							
CCND2	0.022	-1.25	down	0.0001	-1.83	down	0.47 *
NCAPG	0.019	-1.73	down	0.003	-1.58	down	0.47 *
MGMT	0.031	+1.12	up	0.011	+1.32	up	0.53 **
GADD45B	0.669	-1.08	n.r.	0.522	+1.17	n.r	0.68 ***
1 dpn							
SDHB	0.0006	-1.19	down	0.028	-1.30	down	0.71 ***
CCND2	0.711	-1.06	n.r.	0.651	-1.09	n.r.	0.46 *
PPARGC1A	0.745	-1.03	n.r.	0.761	-1.04	n.r.	0.67 ***
188 dpn							
PRKAA1	0.0004	-1.68	down	0.027	-1.40	down	0.77 ***
PRKAA2	0.009	-1.36	down	0.095	-1.25	down	0.10
PPARGC1A	0.126	-1.35	n.r.	0.105	-1.36	n.r.	0.86 ***

CCND2 - cyclin D2; *NCAPG* - non-SMC condensin I complex, subunit G; *MGMT* - O-6-methylguanine-DNA methyltransferase; *GADD45B* - growth arrest and DNA-damage-inducible, β ; *SDHB* - succinate dehydrogenase complex, subunit B, iron sulfur (Ip); *PPARGC1A* - Peroxisome proliferator activated receptor γ coactivator-1α; *PRKAA1* - 5'-AMP-activated protein kinase, catalytic α1 chain; *PRKAA2* - 5'-AMP-activated protein kinase, catalytic α2 chain *calculated by factorial normalisation on *RPL10* expression values; **p-value of pamn' rho; n.r. - not regulated

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ontogenetic growth is not finished at adult stage in LP offspring in contrast to AP offspring.

Furthermore, the dietary modifications led to regulation of the 'Wnt signaling' pathway, which is involved in various aspects of embryogenesis, including cell differentiation and cell proliferation [38]. Therefore, the observed mRNA expression patterns might contribute to an impaired developmental growth in LP offspring.

Regarding the LP model used in rodents, it has been suggested that the maternal LP diet had an impact on 'insulin signaling' as well as on 'IGF1 signaling' and its binding proteins [30,39-43]. However, in this study no regulation of both 'insulin signaling' and 'IGF1 signaling' was observed in liver tissue of LP porcine offspring, possibly due to maturation-, species-, tissue-, sex- and time-dependent regulations.

Transcriptional excursions regarding lipid-, energy- and N-metabolism

During postnatal development a remarkable number of diet- and stage-dependent transcriptionally regulated pathways were related to lipid metabolism, including 'fatty acid metabolism', 'fatty acid elongation in mitochondria', 'synthesis and degradation of ketone bodies', 'bile acid synthesis' and 'biosynthesis of steroids'. These findings suggest that pathways associated to lipid metabolism are part of postnatal adaptive responses to the prenatal nutritional environment in LP offspring. Consistent with these observations, genes associated with lipid metabolism were found to be altered at pre- and postnatal stages in rodents, where the LP model was studied intensively [44-49]. Furthermore, the transcriptional adaptations may have consequences for the offspring's phenotype, including alterations of plasma parameters [44-46,50], hepatic histology [46] and body composition [47].

According to the longitudinal study design the 'AMPK (AMP-dependent activated kinase) signaling' was found to be regulated throughout ontogenesis in AP offspring only. 'AMPK signaling' is a metabolic pathway which is involved in the regulation of the lipid and energy metabolism in mammalian cells [51-56]. In particular, the cellular energy sensor AMPK is essential for a proper mitochondrial activity and metabolic health citeTowler2007. Therefore, the up-regulation of 'AMPK signaling' in developmental periods I, II and III in AP offspring accounts for metabolic health in AP offspring, but not in LP offspring. On the one hand, this matter may be related to mitochondrial impairments which may happen due to the LP diet, as has been suggested previously [57,58]. On the other hand, it might be associated to the transcriptional excursions within the lipid metabolism in LP offspring. Moreover, the expression of the catalytic subunits of AMPK, *PRKAA1* and *PRKAA2* was found to be decreased in LP offspring compared to AP offspring at stage 188 dpn, which may contribute to an imbalanced cellular metabolism. Taken together, the observed regulations were seen as a clue for impairments in energy metabolism in LP offspring during ontogenesis.

Furthermore, the diet-dependent transcriptional excursions revealed an impact on N-metabolism. In particular, the degradation of essential, branch-chained amino acids like valine, leucine and isoleucine was found to be regulated, which may illustrate the LP offspring's efforts to mimic prenatal experiences in terms of dietary supply during postnatal development in the face of richer conditions. Although essential amino acids are needed for growth, anabolic and developmental processes, transcriptional levels of genes leading to degradation of those dietary valuable compounds is forced during postnatal development in LP offspring.

Transcriptional excursions regarding stress and immune response

The biological effect of glucocorticoids is modulated by glucocorticoid receptors. By means of succeeding direct and indirect interactions of downstream target genes, mRNA expression regarding metabolic, behavioural, cardiovascular and immune functions is modulated. In rodents, many studies reported an impact of maternal low protein diets on expression and affinity of the glucocorticoid receptor and its signaling molecules [8,48,59-61]. Consistently, in porcine offspring a diet-dependent regulation of 'glucocorticoid receptor signaling' was also found. Therefore, expression profiles at weaning and adult stages point to an alarm alert (activated defence) in LP offspring. Furthermore, genes associated with the complement system were found to be regulated in a diet-dependent manner at stage 28 dpn. Obviously, at least a part of the innate immune system is impaired when LP offspring is faced with a pre-biotical stress like weaning. Consistent with this finding, expression of genes associated with an impaired immunity were found to be altered in offspring of protein malnourished dams [62,63].

Conclusions

In conclusion, the longitudinal survey of the hepatic transcriptomic response in offspring of dams fed either a gestational low protein diet (LP, 6% CP) or an adequate protein diet (AP, 12% CP) at prenatal (94 dpc), perinatal (1 dpn), juvenile(28 dpn), and adult (188 dpn) ontogenetic stages revealed acute short-term and delayed long-term modulations. The changes in gene expression were not persistent in terms of consistent differential expression of genes at all stages. However, genes related to cell cycle and cellular turnover were

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differentially expressed at all stages except stage 1 dpn. This stage appeared as a phase of recovery of the activity of genes. Differential expression of genes related to lipid, ketone body, and amino acid metabolism indicate that the offspring of both groups uses different metabolic directions in response to identical nutritional condition during postnatal life.

Methods

Animals and sample collection

Animal care and tissue collection processes followed the guidelines of the German Law of Animal Protection and the experimental protocol was approved by the Animal Care Committee of the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LVL MV/TSD/7221.3-1.1-006/04; LALLF M-V/TSD/7221.3-1.2-05/06; LALLF M-V/TSD/7221.3-1.2-013/06). The animal experiment was performed as described [15]. Briefly, at insemination German Landrace primiparous sows (n = 42) were randomly assigned to either a low protein diet (LP) with 6% (w/w) crude protein or an adequate protein diet (AP) containing 12% crude protein. Diets were formulated to be isocaloric (13.6 MJ ME/kg on average) [15]. Tissue sampling included offspring of these sows at one prenatal (94 dpc) and three postnatal (1, 28, 188 dpn) time points (Figure 3).

At 94 dpc of gestation, a subset of eight sows per dietary group was subjected to Caesarean section (EXP1). Sows were anaesthetised as described [15]. This experiment was performed over 5 replicates. Eight viable fetuses per sow were collected starting at the tip of the left uterus horn and alternating between left and right horn. Fetuses were killed by i.v. injection of T61 in the V. cava cranialis and liver samples were immediately collected (approximately 500 mg), frozen in liquid nitrogen, and stored at -80°C until analysis. Fetuses originated from litters of at least 11 viable piglets. Fetuses of LP-fed dams showed a numerically lowered weight compared to AP fetuses at 94 dpc (LP: 661 ± 115 g, and AP: 711 ± 118 g, respectively; $P = 0.12$; n = 32). The smallest and the heaviest fetus within one litter were selected for transcriptome analysis.

In the second experiment (EXP2) offspring selected for the postnatal time points was born to primiparous sows after prostaglandin induction of parturition as described [15] and farrowed after mean pregnancy duration of 115 days. This experiment was conducted over 8 replicates and offspring of a subset of 4 sows (2 per diet per replicate) with a minimum of 11 live born piglets (median litter size = 13) were used. At birth 10 piglets in each litter were distributed over three time points (1, 28, 188 dpn). For the microarray analyses, 8 sib pairs (light and heavy piglet from one litter) which were balanced for sex (all stages) and discordant for weight (light and heavy piglet; stages 94 dpc and 1 dpn only) were chosen per stage and diet.

Mean birth weights of LP newborn piglets used for microarray analyses were numerically lower than birth weights of AP offspring (LP: 1.21 ± 0.30 kg, AP: 1.36 ± 0.31 kg, $P = 0.08$, n = 32). Including all piglets derived from the complete experiment, LP offspring was growth-restricted compared to AP offspring, but litter size did not differ [15]. Thirty-six hours after birth, the lightest and the heaviest piglet within one litter were killed by i.m. injection of 1.25 mg propionyl-promazine (0.2 ml Combelen, Bayer AG, Leverkusen, Germany) and 50 mg ketamine (Ursotamin, Serumwerk Bernburg AG, Germany). Samples were immediately collected from lobus sinister hepaticus (approximately 500 mg), frozen in liquid nitrogen, and stored at -80°C until analysis.

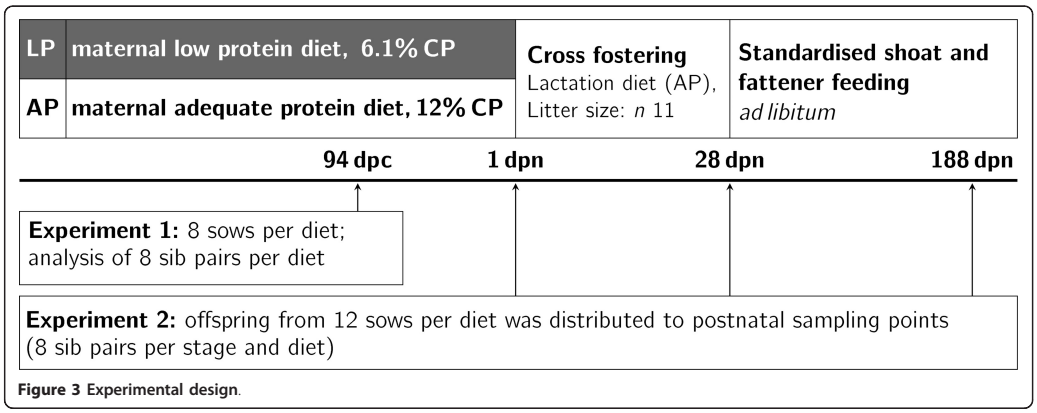

Figure 3 Experimental design.

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The remaining piglets were cross-fostered to non-experimental sows of 2nd to 4th parities, which were on the AP diet during gestation. All sows were fed AP lactation diets. Litter size during suckling was standardised to 11 piglets per sow. Male piglets were castrated at d 4 of age. From weaning (28 dpn) to slaughter (188 dpn), all piglets were individually reared. They had free access to standard diets formulated for post-weaning (d 29 to d 76), growing (d 77 to d 105) and finishing periods [64]. At 28 dpn and 188 dpn of age, pigs were weighed after an overnight fast and killed by electronarcosis followed by exsanguination in the experimental slaughterhouse of FBN. Among the animals used for the microarray expression analysis, weaners of the LP group showed numerically but not significantly lower body weight than the AP group, (LP: 7.24 ± 1.56 kg, AP: 7.59 ± 2.14 kg, P = 0.30, n = 32). At 188 dpn the body weight was slightly but significantly reduced in LP compared to AP (LP: 123.19 ± 9.15 kg, AP: 131.55 ± 15.11 kg, P - 0.03; n - 32). For all animals of the experiment no significant differences in body weight were observed at 28 and 188 dpn [15,16]. Liver tissue was immediately collected from lobus sinister hepaticus, frozen in liquid nitrogen, and stored at - 80°C until use for RNA isolation.

RNA isolation, target preparation and hybridisation

According to the manufacturer's protocol total RNA from individual liver samples was isolated using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany). After DNase treatment a column-based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) was done. The RNA samples were visualised on 1% agarose gels containing ethidium bromide to check RNA integrity. RNA was quantified by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). To ensure the absence of a DNA contamination within the isolated RNA a PCR amplification with the porcine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was done (Forward primer: aagcagggatgatgttctgg; Reverse primer: atgcctcctgtaccaccaac). All RNA samples were stored at -80°C until downstream analysis was performed. For the microarray experiments individual biotin-labeled cRNA was synthesised by the Gene Chip 3' Express Kit (Affymetrix, Santa Clara, CA, USA). The cRNA was fragmented (≈ 100 bp) and hybridised on Affymetrix GeneChip® porcine Genome Array. After staining and washing steps the arrays were scanned (Affymetrix, Santa Clara, CA, USA).

Data analysis

The bioinformatic analysis was done in R [65]. Firstly, a quality control was performed. At 94 dpc 15 AP-samples met the appropriate quality control criteria (94 dpc-LP: 16; 1 dpn-AP: 15; 1 dpn-LP: 14; 28 dpn-AP: 14; 28 dpn-LP: 15; 188 dpn-AP: 16; 188 dpn-LP: 16 samples). Samples were GC-RMA normalised (Log2). The MAS5 algorithm was used to skip those transcripts which were expressed in less than 50% of the animals within one dietary group per stage. For a second filtering step standard deviations were calculated for each probe-set over all subsets of arrays of the particular comparisons. Probe-sets with a low standard deviation ($s \leq 0.25$) were discarded, because such transcripts are not likely to be regulated.

Relative changes in mRNA levels were determined using a mixed model analysis, including effects of dietary treatment, sex, mother, birth weight and interaction between diet and sex ($v_{ijkl} = diet_i + sex_j + mother_k\ (diet_i) + weight_l + (dietxsex)_{ij} + error_{ijkl}$). P-values (significance set at $P \leq 0.05$) for each stage were converted to a set of q-values ($q \leq 0.25$) using the algorithm proposed by Storey and Tibshirani [66]. In general, results are given for the comparisons in the direction of LP vs. AP; thus 'up-regulation' or 'increased expression' indicates higher expression in LP than in AP. Analysis of the pathways involved was carried out using Ingenuity Pathway Analysis [67]. The up-to-date annotation of Affymetrix probe-sets to EnsEMBL Sscofa 9 (20,439 of 23,935 annotated probe-sets) was used [18]. All the microarray data is MIAME compliant and the raw data has been deposited in a MIAME compliant database, the National Center for Biotechnology Information Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo (accession numbers: GSE25482 and GSE31191).

Pathway analysis

Gene lists from microarray results were submitted to the manually curated database 'Ingenuity Pathways Analysis' to elucidate putative pathways associated with an altered gene expression in porcine liver. The focus was on those canonical pathways which came up at least once within the top ten regulated pathways within one single analysis. It should be noted here that the interactions presented in the networks are not specific for porcine liver tissue, as the database contains literature from many different research areas.

Quantitative real-time RT-PCR

First-strand cDNA was synthesized from 2μg of total RNA (n = 14 per diet and stage) using random primers and oligo d(T) 13VN in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). In order to survey expression of the liver tissue samples, total transcript levels of selected target and reference genes (Table 5) were quantified by real-time quantitative PCR (qPCR) performed on a LightCycler®480 system using the LightCycler 480 SYBR Green I Master(Roche, Mannheim, Germany). The amplification was conducted in duplicate according to manufacturer's instructions

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Table 5 Primer used to verify microarray experiments in liver tissue by qRT-PCR

Gene name	Probe-set ID	Sequence 5′ - 3′	T(°C)	Size (bp)
CCND2	Ssc.15749.1.S1_at	For AGGAGCAGATTGAGGTCGTG	86	185
		Rev CAACCAGAGAGAAGGAAGGAGA		
NCAPG	Ssc.28512.1.S1_at	For CTTGTAGATTTGACGAGACCA	60	156
		Rev GGCTTTAGTATAGACCCGAAC		
MGMT	Ssc.19639.1.A1_at	For GCAACTACTCGGGAGGAATG	88	171
		Rev CTGCGAACGCTCAGTCTTG		
GADD45B	Ssc.14764.1.A1_at	For GGACTTAGACTTTGGGACTTG	60	140
		Rev GTAAGCCTCCCATCTCTCTT		
SDHB	Ssc.8939.1.S1_at	For GCAGGACCCGTTCTCTCTGT	60	170
		Rev GGTTACAGTCACGTTAGGTTGG		
PPARGC1A	Ssc.16864.1.S1_at	For GTAAATCTGCGGGATGATGG	60	208
		Rev TGGTGGAAGCAGGATCAAAG		
PRKAA1	Ssc.8107.1.A1_at	For TTGTTAATTTCATAAACTTTGCTTC	60	193
		Rev GTGCAGCCTTGACATACTC		
PRKAA2	Ssc.16257.1.S1_at	For TCTGTAATTCTGTTTTGCCTACGA	60	168
		Rev AGCAAGAAGGTGATGCCAAG		
RPL10*	Ssc.9130.1.A1_at	For CTGTGTTCGTCTTTTCTTCC	60	199
		Rev TCATCCACTTTTGCCTTCT		

CCND2 - cyclin D2; *NCAPG* - non-SMC condensin I complex, subunit G; *MGMT* - O-6-methylguanine-DNA methyltransferase; *GADD45B* - growth arrest and DNA-damage-inducible, β ; *SDHB* - succinate dehydrogenase complex, subunit B, iron sulfur (Ip); *PPARGC1A* - Peroxisome proliferator activated receptor γ coactivator-1α; *PRKAA1* - 5′-AMP-activated protein kinase, catalytic α1 chain; *PRKAA2* - 5′-AMP-activated protein kinase, catalytic α2 chain; *RPL10* - Ribosomal protein 10; * house keeping gene.

using 10 μM of each primer. Reactions were performed in a final volume of 10μl using 5.0μl of LightCycler 480 SYBR Green I Master (Roche), 2.0 μl of *Aqua dest*., 10 μM (0.5 μl) of each primer (Table 5) and 40 ng (2 μl) cDNA. The temperature profiles comprised an initial denaturation step at 95°C for 10 min and 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 60° C for 10 s and extension/fluorescence acquisition at 72° C for 15 s. For all the assays threshold cycles were converted to copy numbers using a standard curve generated by amplifying serial dilutions of an external PCR standard (10^7 - 10^2 copies). 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, 28 individual liver mRNA samples were analyzed in duplicate per stage. Data were analyzed using the PROC MIXED, including effects of diet, sex, mother, birth weight and interaction between diet and sex (SAS version 9.1; SAS Institute, Cary, NC). Differences were considered significant at $P \leq 0.05$.

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Author details
[1]Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. [2]Research Unit Physiology of Nutrition, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. [3]Research Group Functional Genomics, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany.

Authors' contributions
Conceived and designed the experiments: KW CCM SP. Performed the experiments: MO EM CCM SP KW. Analyzed the data: MO EM SP KW. Contributed reagents/materials/analysis tools: CCM KW SP EM. Wrote the paper: MO KW. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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2.4 A gestational high protein diet affects the abundance of muscle transcripts related to cell cycle regulation throughout development in porcine progeny.

Michael Oster, Eduard Murani, Cornelia C. Metges, Siriluck Ponsuksili and Klaus Wimmers

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Conceived and designed the experiments: KW CCM SP. Performed the experiments: MO EM CCM SP KW. Analyzed the data: MO EM SP KW. Contributed reagents/materials/analysis tools: CCM KW SP EM. Wrote the paper: MO KW.

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A Gestational High Protein Diet Affects the Abundance of Muscle Transcripts Related to Cell Cycle Regulation throughout Development in Porcine Progeny

Michael Oster[1], Eduard Murani[1], Cornelia C. Metges[2], Siriluck Ponsuksili[3], Klaus Wimmers[1]*

1 Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, **2** Research Unit Nutritional Physiology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, **3** Research Group Functional Genomics, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Abstract

Background: In various animal models pregnancy diets have been shown to affect offspring phenotype. Indeed, the underlying programming of development is associated with modulations in birth weight, body composition, and continual diet-dependent modifications of offspring metabolism until adulthood, producing the hypothesis that the offspring's transcriptome is permanently altered depending on maternal diet.

Methodology/Principal Findings: To assess alterations of the offspring's transcriptome due to gestational protein supply, German Landrace sows were fed isoenergetic diets containing protein levels of either 30% (high protein - HP) or 12% (adequate protein - AP) throughout their pregnancy. Offspring muscle tissue (*M. longissimus dorsi*) was collected at 94 days post conception (dpc), and 1, 28, and 188 days post natum (dpn) for use with Affymetrix GeneChip Porcine Genome Arrays and subsequent statistical and Ingenuity pathway analyses. Numerous transcripts were found to have altered abundance at 94 dpc and 1 dpn; at 28 dpn no transcripts were altered, and at 188 dpn only a few transcripts showed a different abundance between diet groups. However, when assessing transcriptional changes across developmental time points, marked differences were obvious among the dietary groups. Depending on the gestational dietary exposure, short- and long-term effects were observed for mRNA expression of genes related to cell cycle regulation, energy metabolism, growth factor signaling pathways, and nucleic acid metabolism. In particular, the abundance of transcripts related to cell cycle remained divergent among the groups during development.

Conclusion: Expression analysis indicates that maternal protein supply induced programming of the offspring's genome; early postnatal compensation of the slight growth retardation obvious at birth in HP piglets resulted, as did a permanently different developmental alteration and responsiveness to the common environment of the transcriptome. The transcriptome modulations are interpreted as the molecular equivalent of developmental plasticity of the offspring that necessitates adaptation and maintenance of the organismal phenotype.

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* E-mail: wimmers@fbn-dummerstorf.de

Introduction

Intrauterine growth retardation (IUGR), i.e., impaired prenatal growth and development, can be caused by an adverse nutritional intrauterine environment characterized by limited or excess protein supply during pregnancy [1,2]. IUGR is a major concern in animal breeding, because of its negative impact on production [3]. Low birth weight piglets grow slower and are prone to impaired carcass and meat properties, including higher drip losses, higher body fat contents, lower muscle mass, lower glycogen reserves and lower tenderness scores when compared with their high birth weight littermates [4–6]. Muscle tissue of low birth weight piglets is hypothesized to develop increased hypertrophy due to reduced myofiber proliferation, which might affect fat deposition and energy metabolism [5]. Therefore, cellular growth and regulation of the cell cycle may be central in the underlying molecular mechanism governing the intrauterine adaptive response to adverse environmental conditions ("fetal programming").

Fetal programming has recently come under heavy investigation. Evidence indicates that diet-dependent permanent consequences on the phenotype involve sophisticated modulations of the gene expression machinery, leading to a diet-specific tuning of the transcriptome in rodents [7,8], pigs [9] and cattle [10]. Notably, it was shown that long-term modulations of gene expression will also take place due to adverse feeding regimes at crucial prenatal time periods, e.g. around conception [11] and early gestation [12]. However, modulation of the transcriptome occurs in response to exogenous effects; such changes may induce and represent adaptive processes to maintain homoeostasis and force the expression of the organismal phenotype within physiological norms. In fact, in our previous work, the progeny of sows that

received either high protein levels or adequate protein levels during pregnancy only showed slight divergences in birth weight and body composition at birth [2,13]. At later stages offspring of both groups became similar in terms of organismal phenotype, including body weight, body composition, and cellularity of muscle and adipose tissue [13,14]. Monitoring the transcriptome at different developmental stages in the context of fetal programming, i.e., variable conditions at prenatal time points, can uncover mechanisms behind prenatal events that affect postnatal development.

We have previously shown that, depending on the gestational diet, the expression profile of a central metabolic organ like liver is affected at both prenatal and postnatal stages, specifically as an altered responsiveness of energy and nutrient-sensing pathways [9]. Here, we focus on muscle tissue; muscle represents the largest peripheral consumer of and storage hub for energy and nutrients, contributes to the species-typical shape of the body, and is a main agricultural product for human consumption when muscle becomes meat. We aimed to identify molecular pathways with relevance to the fetal initiation of postnatal growth and development in pigs. Pregnant German Landrace gilts were fed isoenergetic diets containing either adequate or excess levels of protein at the expense of carbohydrate supply. Gene expression profiles of their fetuses and offspring were investigated in a longitudinal experimental design. Transcriptome analyses indicate that maternal protein supply and/or shortage of exogenous carbohydrates triggers a programming of the offspring's genome characterized by the adjustment of molecular routes related to cell cycle regulation and proliferation. These modulations contribute to the maintenance of the overall organismal phenotype of the offspring independent of prenatal nutritional experiences.

Results

To develop an index of genes responsive to gestational diets and to characterize molecular pathways and mechanisms related to fetal programming, we analysed diet-dependent expression patterns in a longitudinal holistic study of muscle tissue collected from progeny of sows fed either isoenergetic high-protein (HP; 30% crude protein; protein: carbohydrate ratio 1:1.3) or adequate-protein diets (AP; 12% crude protein; protein: carbohydrate ratio 1:5). Offspring muscle gene expression was determined at one prenatal and three postnatal stages using porcine 24 k microarrays. Transcriptional alterations of genes and resulting metabolic pathways were identified according to the variance component diet × stage. Therefore, transcriptional shifts between HP and AP animals at each developmental stage (Figures 1 and 2, vertical arrows) as well as transcriptional shifts between adjacent stages among the experimental groups (Figures 1 and 2, horizontal arrows) were considered.

In comparing HP vs. AP, 15,738 probe sets overall were found to be expressed in muscle tissue according to MAS5 analysis. Further filtering based on the variability of expression of probe sets revealed 14,267 probe sets for further analysis. These probe sets represent 9,666 genes according to the recent annotation [15].

Comparisons between HP and AP within stages

Expression profiles in HP and AP offspring were compared within each developmental stage. At 94 dpc, 465 probe sets were significantly different between HP and AP fetuses (227 HP≻AP; Figure 1). Ingenuity Pathway Analysis indicated enrichment of molecular routes related to cyclin and cell cycle regulation, G1/S checkpoint regulation, and actin cytoskeleton signaling and organization of filaments, which were found to be more abundant in HP offspring (Table 1, Figure 2). In perinatal piglets (1 dpn), 267 probe sets differed between HP and AP offspring (188 HP≻AP). The abundance of mRNA of genes associated with organisation of filaments was found to be increased in HP offspring. Remarkably, in infant piglets at weaning (28 dpn), no probe sets differed between HP and AP offspring. In young adult pigs (188 dpn) 20 probe sets were significantly different between HP and AP offspring (7 HP≻AP). Notably, probe sets representing two genes (SMAD4, CDC27) related to canonical pathways of G1/S checkpoint regulation and mitotic roles of Polo-like kinase were less abundant in HP offspring at 188 dpn.

Differences of longitudinal developmental changes of transcript abundance among HP and AP offspring

Expression patterns of two adjacent developmental stages within each dietary group were compared to determine transcripts showing an altered abundance between stages ($P \leq 0.05$) (Figure 1). Resulting gene lists were compared between HP and AP offspring at the particular stage. The intersection of commonly altered genes between those comparisons represent genes that show the same shift of abundance along stages in both dietary groups and thus likely belong to physiological maturation processes. Therefore, the analysis focused on those transcripts whose change of abundance between two consecutive stages (period I: 94 dpc-1 dpn; period II: 1 dpn-28 dpn; period III: 28 dpn-188 dpn) was limited to either the HP or the AP group. Those represent genes displaying diet-dependent longitudinal transcriptomic alterations.

Between fetal and perinatal stages (period I), 1,750 (605 1 dpn≻94 dpc in AP) probe sets were altered in level or direction of change of transcript abundance in the AP group compared to the HP group. At 1 dpn, expression of genes associated with RAN signaling and IGF1 signaling was increased; expression of genes participating in organisation of filaments was decreased (Table 2). During the same period, 1,702 probe sets in HP offspring were altered (770 1 dpn≻94 dpc in HP) when compared to the AP group. mRNA levels of genes associated with oxidative phosphorylation, purine metabolism, pyrimidine metabolism, and synthesis and degradation of ketone bodies were higher at 1 dpn than at 94 dpc in HP offspring. Conversely, genes participating in glucocorticoid receptor signaling, cyclin and cell cycle regulation, G1/S checkpoint regulation, growth hormone signaling, mitotic roles of Polo-like kinase, and IGF1 signaling were less abundant in HP perinatal piglets.

When comparing perinatal and infant piglets (period II), expression of 1,864 probe sets was altered in a different manner in AP offspring than in HP offspring. Of these, 900 probe sets were upregulated and 964 probe sets were downregulated by 28 dpn. Genes participating in the organisation of filaments as well as in purine and pyrimidine metabolism were more highly expressed, while genes associated with fatty acid elongation in mitochondria, RAN signaling, and oxidative phosphorylation were decreased during that period in AP offspring. In the same period, 2,130 probe sets exhibited variation that was specific to HP offspring. Of these, 870 probe sets had an increased mRNA level at 28 dpn, including genes involved in G2/M DNA damage checkpoint regulation, mitotic roles of Polo-like kinase, RAN signaling, and oxidative phosphorylation. Decreased mRNA expression was detected for genes associated with the organisation of filaments.

When infant and young adult pigs (period III) were compared, 1,556 probe sets differed significantly (937 188 dpn≻28 dpn in AP) in AP offspring. Increased expression in adult AP offspring was observed for genes participating in AMPK signaling, mitotic roles of Polo-like kinase, mTOR signaling, and cyclin and cell cycle regulation. In HP offspring 1,724 probe sets were

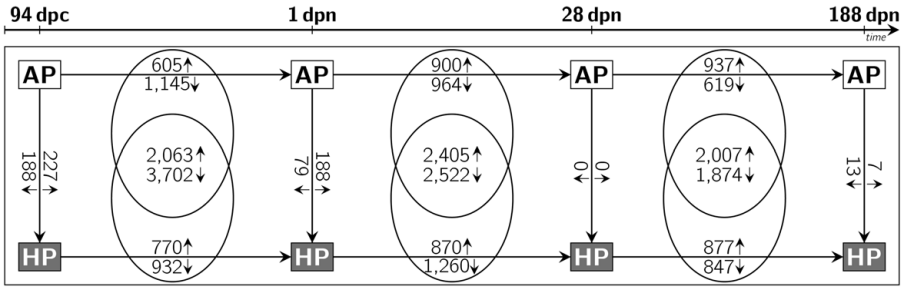

Figure 1. Number of probe sets showing a significantly altered abundance in muscle tissue. The number of altered probe sets between adjacent developmental stages in AP or HP offspring are indicated at horizontal arrows; the number of commonly altered probe sets between stages in AP and HP offspring are indicated at intersections; the number of probe sets showing a different abundance between HP and AP offspring at the same developmental stage are indicated at vertical arrows; small arrows at the numbers indicate a higher or lower probe set abundance, respectively.
doi:10.1371/journal.pone.0034519.g001

differentially expressed (877 188 dpn≻28 dpn in HP). Adult HP offspring had higher expression of transcripts involved in purine metabolism, pyrimidine metabolism, and glucocorticoid receptor signaling. Expression was lower in adult HP offspring for genes involved in oxidative phosphorylation, mitotic roles of Polo-like kinase, and G2/M DNA damage checkpoint regulation.

A comprehensive overview of the pathways observed to be altered between diets (HP vs. AP) and stages (94 dpc; 1, 28, 188 dpn) is depicted in Figure 2.

Discussion

In a longitudinal study covering prenatal, perinatal, juvenile, and adult developmental stages in a porcine model, we analysed muscle gene expression patterns in offspring from sows fed either an isoenergetic high-protein low carbohydrate or adequate control protein diet throughout pregnancy. Relative mRNA abundances were compared between both dietary groups and developmental stages. Variation of transcript abundances at various pre- and postnatal stages in muscle has been shown [16–18]. Accordingly, a longitudinal study was done and evaluated focussing on the interaction of diet x stages in order to consider mechanisms of fetal programming at variable stages-dependent transcriptomic backgrounds. Also impact of IUGR and low birth weight as well as sex on postnatal growth is well documented [19–21]; however, our experiment was designed to balance for sex and body weight among the diet-groups at all stages; accordingly, only a few probe-sets were found exhibiting variable abundance due to sex (24 probe-sets) or weight (8 probe-sets). mRNA expression profiles

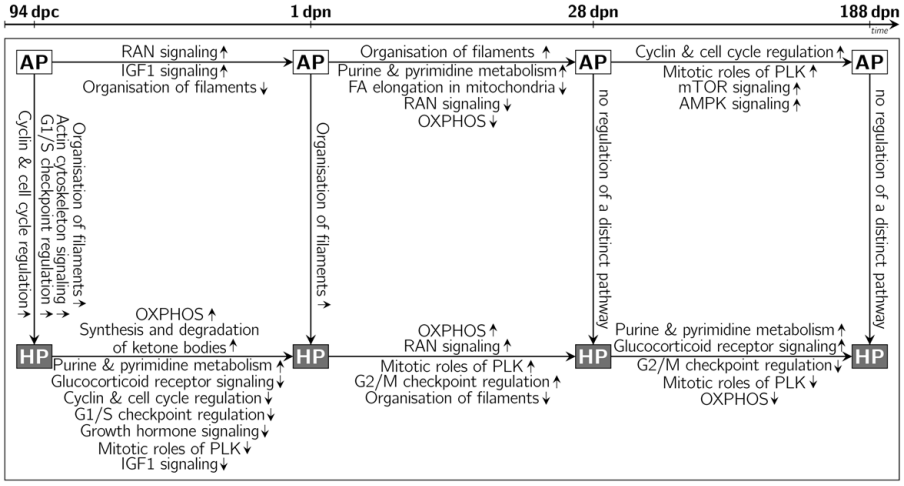

Figure 2. Affected pathways in muscle tissue between developmental stages and diets. Listed pathways between AP stages (white boxes) indicate shifts during development that are not found in HP offspring (black boxes) at the corresponding period. Pathways between HP stages indicate alterations that occur in HP offspring but not in AP offspring in the corresponding period. (Arrows between boxes show direction of comparison; small arrows indicate higher and lower transcript abundance, respectively. OXPHOS, oxidative phosphorylation; PLK, Polo-like kinase; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; IGF1, insulin-like growth factor 1; FA, Fatty acid; RAN, Ras-related nuclear protein).
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Table 1. Functional annotation of muscle transcripts showing altered abundance depending on the dietary group (HP vs. AP) within different developmental stages (Ingenuity Pathway Analysis).

Developmental stage	Affected pathway	Expression	*P* value	No. of genes involved	Genes involved in pathway
94 dpc	Actin cytoskeleton signaling	up	1.02*E-2	7	ACTA1, CDC42, MSN, RDX, SSH2, TTN, VCL
	G1/S checkpoint regulation	up	2.48*E-2	3	CDK6, E2F2, HDAC6
	Cyclin and cell cycle regulation	up	1.19*E-2	4	CDK6, E2F2, HDAC6, PPP2R1A
	Organisation of filaments	up	1.53*E-4	8	COL1A1, COL1A2, COL5A1, LOX, MSN, NCK2, RDX, RHOB
1 dpn	Organisation of filaments	up	1.03*E-7	10	AKAP2, COL1A1, COL1A2, COL5A1, COL5A2, DCN, FN1, LOX, P4HA1, SERPINH1
28 dpn	-	-	-	-	-
188 dpn	-	-	-	-	-

Up and down indicate higher and lower abundance in HP compared to AP, respectively. P-value: significance of association between dataset and IP-pathways; Fischer's exact test.

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between diets revealed significant shifts at prenatal and postnatal stages. Even more distinct differences were observed between dietary groups when considering temporal changes along development. Thus, the maternal diet produced both short- and long-term transcriptional alterations in the offspring. Because offspring were exposed to standard dietary conditions from birth (through fosters) to adult, in both experimental groups the transcriptional alterations were consequences of the maternal protein supply during prenatal development. In particular, a number of cell cycle modulating pathways and energy producing pathways in muscle tissue were affected by diet.

Growth and cell maintenance

In mammals cell division is synchronous with cell growth (reviewed in [22,23]), which is triggered by various factors and functional networks. The fidelity of cell division is monitored in eukaryotic cells by sophisticated mechanisms, including defined checkpoints that initiate cell cycle arrest if processes go awry. Among others, Polo-like kinases (PLK), an evolutionarily conserved family of essential cell cycle regulators, are required at several key points within the cell cycle to ensure entry and exit from mitosis (reviewed in [24,25]). Cell cycle regulators have been shown to exhibit altered expression at both RNA and protein levels due to varied maternal protein diets [26,27]. It can be supposed that altered expression of cell cycle regulators will affect cell growth and proliferation. At the tissue and organ level, this could translate into differences in body composition and organ weights. In fact, in two recent studies we reported that excess maternal protein during pregnancy produced slightly smaller pigs with reduced muscularity [13,14]. Previous work had demonstrated that the number of secondary myofibers present in prenatal skeletal muscle is dependent on the maternal diet [28]. Thus, it appears that maternal diet affects the growth of offspring skeletal muscle prenatally, resulting in some level of IUGR. However, this affect may be transient: we also observed that excess protein supply in sows during pregnancy had only modest effects on the phenotype of juvenile and adult pigs, which only showed slightly lowered muscularity and slight, non-significantly reduced muscle fiber number [13,14]. Interestingly, juvenile offspring of the HP group had a smaller percentage of STO (slow-twitch oxidative) fibers ($P = 0.09$) than juvenile AP offspring [14], suggesting a potential impact of the gestational dietary high protein but low carbohydrate intake on primary muscle fiber development. Judged by the metabolic characteristics of the pregnant dams it is possible that HP sows suffer from metabolic energy deficit due to the need for high rates of ureagenesis and gluconeogenesis triggered by the high protein and low carbohydrate intakes [29]. How this is translated to the fetus is difficult to deduce but additionally might suggest specific differences in amino acid supply in the HP offspring (Metzler-Zebeli et al., manuscript under review Br J Nutr [30]). By 188 dpn, though, there were no differences in fiber type frequencies among the dietary groups [14].

Our findings of altered expression in the molecular routes related to cell cycle and organization of filaments are the transcriptional equivalent of the high developmental plasticity exhibited by the offspring, which ensures maintenance of the overall organismal phenotype by adaptive response of cells of the muscle tissue including satellite cells, fat cells, and fibroblasts. Increased gene expression of cell cycle-associated pathways at 94 dpc may indicate possible compensatory growth mechanisms to counteract the observed IUGR.

In HP offspring, the direction of change of cell cycle and growth-associated pathways was stage-dependent. Within developmental period I for HP offspring, transcripts interacting in various cell cycle and growth-related pathways occurred with lower abundance perhaps reflecting an impaired fetal growth performance due to a potential metabolic energy deficit caused by the gestational HP diet [29] and, therefore, contributing to the observed IUGR at birth [2]. In fact, altered expression of genes related to cell cycle and growth regulating pathways might lead to a lengthened cell cycle and culminate in growth retardation [31]. Our findings seem to indicate that, in this regard, a balancing occurs postnatally. In contrast, AP fetuses in period I showed an increased expression of transcripts associated with cell division, including RAN signaling, a positive key regulator of mitosis (reviewed in [32]). During the same period transcripts related to organization of filaments, including transcripts of collagen genes, were more abundant in HP and remained opposed in abundance to that in AP offspring during early postnatal development. Interestingly, the collagen content of porcine skeletal muscle is associated with intrauterine growth, with small littermates having higher collagen content [33,34].

mRNA expression within developmental period II revealed an increased abundance of transcripts related to cell division-

Table 2. Functional annotation of muscle transcripts showing altered abundance between two developmental stages within either dietary group HP or AP (Ingenuity Pathway Analysis).

Developmental comparison	Diet	Affected pathway	Expression	*P* value	No. of genes involved	Genes involved in pathway
94 dpc vs. 1 dpn	AP	RAN signaling	up	8.64*E-3	3	CSE1L, IPO5, KPNA1
	AP	IGF1 signaling	up	3.97*E-2	6	CSNK2A1, MAP2K1, PRKAR2A, RAF1, SOS1, YWHAG
	AP	Organisation of filaments	down	1.26*E-2	13	B4GALT7, BGN, CNP, COL1A2, COL2A1, COL5A1, EVL, FGF2, FNBP1, LOX, MTSS1, TGFB1, TNXB
	HP	Purine metabolism	up	3.19*E-4	21	ADCY3, ATP13A2, ATP6V0B, BCKDHA, BCKDHB, ENTPD4, GMPR, ITPA, MAD2L2, NME1, NME2, NME3, NUDT2, POLD4, POLR1C, POLR2G, POLR2I, POLR2L, PSMC1, RRM2B, RUVBL1
	HP	Pyrimidine metabolism	up	1.19*E-5	16	ENTPD4, ITPA, MAD2L2, NME1, NME2, NME3, NUDT2, POLD4, POLR1C, POLR2G, POLR2I, POLR2L, RPUSD1, RPUSD2, RPUSD4, RRM2B
	HP	Oxidative phosphorylation	up	1.24*E-2	10	ATP6AP1, ATP6V0B, ATP6V0E2, COX6B1, NDUFA8, NDUFB7, NDUFB9, NDUFS3, RPUSD1, TNNI2
	HP	Synthesis and degradation of ketone bodies	up	4.43*E-3	3	ACAA1, ACAT2, BDH1
	HP	Glucocorticoid receptor signaling	down	1.27*E-4	24	CCL2, FOS, GTF2E2, GTF2H3, JUN, NCOA1, NCOA2, NCOA3, NCOR1, PIK3C3, PIK3R3, POLR2D, PPP3CB, PRKACB, RRAS2, SERPINE1, SHC1, SMAD3, STAT1, SUMO1, TBP, TGFB2, TGFBR2, TRAF6
	HP	Cyclin and cell cycle regulation	down	8.09*E-5	12	ATR, CCNA2, CDKN1B, E2F2, HDAC3, HDAC6, PPP2CB, PPP2R1A, PPP2R1B, PPP2R5E, RB1, TGFB2
	HP	G1/S checkpoint regulation	down	1.86*E-3	8	ATR, CDKN1B, E2F2, HDAC3, HDAC6, RB1, SMAD3, TGFB2
	HP	Growth hormone signaling	down	1,71*E-3	9	FOS, GHR, IGF1, IGFBP3, PIK3C3, PIK3R3, RPS6KA3, RPS6KA5, STAT1
	HP	Mitotic roles of Polo-like kinase	down	3.51*E-2	6	CDC27, PLK4, PPP2CB, PPP2R1A, PPP2R1B, PPP2R5E
	HP	IGF1 signaling	down	8.90*E-5	13	FOS, IGF1, IGFBP3, IGFBP5, JUN, PIK3C3, PIK3R3, PRKACB, PTPN11, RRAS2, SHC1, YWHAB, YWHAE
1 dpn vs. 28 dpn	AP	Purine metabolism	up	7.50*E-3	21	ACIN1, ATP11B, ATP13A2, ATP5G2, ATP6V0B, CHRAC1, CILP, DDX19B, DGUOK, NME3, PDE2A, PDE5A, POLG, POLR2D, POLR2E, POLR2F, POLR2J, POLR3K, PRPSAP2, PSMC5, RFC3
	AP	Pyrimidine metabolism	up	5.95*E-3	13	CHRAC1, CTPS2, NME3, POLG, POLR2D, POLR2E, POLR2F, POLR2J, POLR3K, RFC3, RPUSD1, RPUSD4, UCK1
	AP	Organisation of filaments	up	5.74*E-3	12	AKAP2, COL1A1, COL1A2, COL5A2, CRYAA, DBN1, DCN, FN1, FNBP1, PDLIM3D, SERPINH1, TGFB1
	AP	Fatty acid elongation in mitochondria	down	4.39*E-4	5	ACAA2, AUH, HADH, HSD17B4, PECR
	AP	RAN signaling	down	2.49*E-2	3	IPO5, RAN, XPO1
	AP	Oxidative phosphorylation	down	1.54*E-9	25	ATP5B, ATP5C1, ATP5F1, ATP5J, ATP6V1A, ATP6V1B2, ATP6V1C1, ATP6V1H, COX15, COX17, COX6C, COX7C, NDUFA1, NDUFA9, NDUFAB1, NDUFB1, NDUFB3, NDUFB5,NDUFC1, NDUFS2, NDUFV2, PPA2, SDHA, UQCR11, UQCRB
	HP	G2/M DNA damage checkpoint regulation	up	2.63*E-4	8	ATR, CCNB1, CCNB2, PTPMT1, RPRM, WEE1, YWHAE, YWHAG
	HP	Mitotic roles of Polo-like kinase	up	4.95*E-4	9	ANAPC1, ANAPC5, CCNB1, CCNB2, PLK4, PPP2R3A, PTTG1, STAG2, WEE1
	HP	Oxidative phosphorylation	up	2.23*E-3	14	ATP5A1, ATP5O, ATP6V1A, ATP6V1E1, COX7B, FAM63B, IP6K2, NDUFB6, NDUFS1, NDUFS4, NDUFV2, PPA1, PPA2, UHRF1BP1
	HP	RAN signaling	up	2.88*E-3	4	CSE1L, IPO5, TNPO1, XPO1
	HP	Organisation of filaments	down	1.46*E-3	16	ARHGEF2, BGN, COL1A1, COL1A2, COL2A1, COL5A1, DBN1, DCN, EVL, FAT1, FES, LOX, MARK4, NUMA1, PPP1R9AD, SIRPA
28 dpn vs. 188 dpn	AP	AMPK signaling	up	1.10*E-2	11	PIK3R1, PPP2CA, PPP2CB, PPP2R2A, PPP2R5A, PPP2R5E, PRKAA1, PRKAA2, PRKAB2, PRKACB, SMARCA2
	AP	Mitotic roles of Polo-like kinase	up	1.56*E-3	8	ANAPC11, HSP90AA1, PPP2CA, PPP2CB, PPP2R2A, PPP2R5A, PPP2R5E, STAG2

Table 2. Cont.

Developmental comparison	Diet	Affected pathway	Expression	*P* value	No. of genes involved	Genes involved in pathway
	AP	mTOR signaling	up	9.70*E-4	14	EIF3A, EIF4B, NAPEPLD, PIK3R1, PPP2CA, PPP2CB, PPP2R2A, PPP2R5A, PPP2R5E, PRKAA1, PRKAA2, PRKAB2, RHOQ, RICTOR
	AP	Cyclin and cell cycle regulation	up	6.40*E-4	10	ATR, GSK3B, HDAC2, PPP2CA, PPP2CB, PPP2R2A, PPP2R5A, PPP2R5E, RAF1, TGFB2
	HP	Purine metabolism	up	8.46*E-3	20	AMPD3, ATF7IP, ATP6V0E1, ATP6V1G2, DDX19B, EIF2AK4, MAD2L2, MPP1, NME6, NT5C3, POLG, POLR1A, POLR2C, POLR2F, PPAT, PSMC1, PSMC3, PSMC5, RFC3, VCP
	HP	Pyrimidine metabolism	up	2.36*E-2	11	CMPK1, EIF2AK4, MAD2L2, NME6, NT5C3, NXN, POLG, POLR1A, POLR2C, POLR2F, RFC3
	HP	Glucocorticoid receptor signaling	up	2.13*E-2	16	A2M, AGT, CXCL3, EP300, HSP90AA1, MAP3K14, NCOA2, NCOR2, NR3C1, PIK3C3, POLR2C, POLR2F, SMAD3, STAT5B, TAT, TSC22D3
	HP	Oxidative phosphorylation	down	1.35*E-3	14	COX15, COX6A1, COX7C, IP6K2, NDUFA2, NDUFB3, NDUFB5, NDUFB6, NDUFB10, NDUFS3, NDUFS4, NDUFS6, PPA2, UQCRB
	HP	Mitotic roles of Polo-like kinase	down	2.30*E-2	6	CCNB2, CDK1, PLK4, PRC1, PTTG1, WEE1
	HP	G2/M DNA damage checkpoint regulation	down	2.25*E-2	5	CCNB2, CDK1, CKS1B, WEE1, YWHAZ

Up and down indicate higher and lower abundance in later compared to earlier stages, respectively. P-value: significance of association between dataset and IP-pathways; Fischer's exact test.

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associated pathways in HP offspring, reflecting a kind of compensation that results in the absence of transcriptional differences at 28 dpn. These transcriptional changes parallel development at the organismal level, with newborns of the HP group having a significantly lower birth weight and lower body fat content than newborns of the control group. However, neither body weight or body composition nor cellularity of muscle and adipose tissue of weaning piglets at 28 dpn showed any differences [13].

Transcriptional alterations regarding energy metabolism and lipid metabolism

Recent studies have demonstrated that metabolic health is highly dependent on mitochondrial integrity, including proper energy production [35–37]. For developmental and metabolic processes, effective generation of ATP is required, which occurs most efficiently via oxidative phosphorylation (OXPHOS) in mitochondria [38]. In rodents and pigs mitochondria are sensitive, at the transcriptional level, to prenatal dietary modifications [9,39,40] and the maternal metabolic status [41]. Here, we consistently observed that a maternal HP diet led to increased mRNA expression levels of OXPHOS-related genes in muscle tissue, which may suggest a transiently lower energy status or higher energy demands leading to an adaptive upregulation of processes leading to ATP synthesis. Interestingly, the expression pattern of genes related to OXPHOS was biphasic across temporal development in HP offspring. Increased levels of OXPHOS-associated genes within developmental periods I and II may indicate increased metabolic activity as a postnatal short-term response in muscle tissue of HP offspring as a result of an exposure to energy deficient intrauterine environment. Therefore, after the prenatal 'steady state' in terms of metabolic activity induced by the maternal HP diet, compensatory mechanisms may occur at early postnatal stages in HP offspring. This phenomenon reflects a high developmental plasticity of HP offspring. However, at adulthood decreased expression levels of genes related to OXPHOS may account for alterations in mitochondrial activity in HP offspring. This is consistent with findings in IUGR rats, which showed an impaired oxidative phosphorylation as adults [42]. Interestingly, within developmental period III a higher abundance of transcripts related to AMPK (AMP-dependent activated protein kinase) signaling was observed in AP offspring. Because AMPK is a potent activator of mitochondrial metabolism [43], the observed expression pattern may account for metabolic health in AP offspring but not in HP offspring at the adult stage. Taken together, these findings suggest impairments in energy metabolism in muscle tissue of HP offspring along development.

Skeletal muscle is the main peripheral tissue functioning in fatty acid oxidation. However, at the transcript level only a few metabolic pathways related to lipid metabolism were altered in muscle tissue in response to diet. This finding supports the assumption that lipid metabolism-related genes are targets of fetal programming in liver tissue [9] rather than in peripheral tissues like skeletal muscle.

Transcriptional alterations regarding stress response

Biological effects of glucocorticoids, including metabolic, behavioural, cardiovascular, and immune functions, are transmitted via glucocorticoid receptors and related downstream signaling molecules. In rodents maternal diet affects mRNA expression of the glucocorticoid receptor and its signaling molecules [44–46]. Consistently, a diet-dependent alteration in glucocorticoid receptor signaling was also found in porcine offspring [9]. Our similar finding of higher abundance of transcripts related to glucocorticoid receptor signaling within developmental period III may indicate that adult HP animals were in a kind of alarm state with possible side effects on health and metabolism.

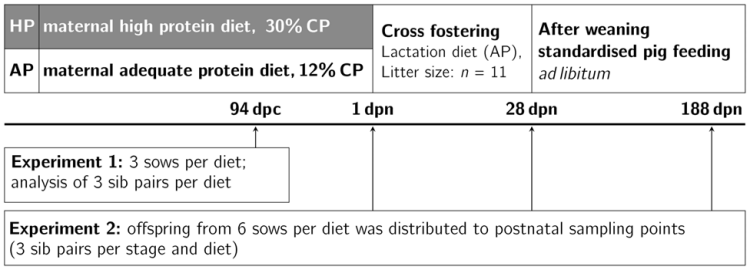

Figure 3. Experimental design. Fetuses and offspring of divergently fed sows were collected at 4 developmental stages. Fetuses were derived from 3 sows per dietary group. Offspring were full sibs of six litters per dietary group collected at 3 consecutive postnatal stages; HP = high protein, CP = crude protein, AP = adequate protein.
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Conclusions

The analysis of longitudinal changes of transcript abundance in skeletal muscle in response to gestational diets with differing protein:carbohydrate ratios revealed both short- and long-term effects. According to expression profiles of HP offspring, alterations of the transcriptome relevant to pathways of growth and cell cycle regulation were modified in response to diet. These alterations might be related to both IUGR and postnatal compensatory effects. Given that dietary effects on the organismal phenotype in terms of body weight, body composition, and cellularity of muscle and adipose tissue were very modest and only transient, the observed transcriptional alterations represent adaptive processes. In liver modulation of energy producing and sensing pathways was observed [9]; in skeletal muscle, however, shifts in molecular routes related to cell cycle are predominant throughout development. Also the temporal sequences of shifts differ between the central metabolic organ, the liver, and the peripheral tissue of muscle. In muscle, differences of the transcriptomes of HP and AP offspring are highest at 94 dpc and 1 dpn, i.e., time points that are important to myogenesis. At later stages the differences observed in longitudinal shifts lead to a minimization of differences between AP and HP offspring phenotypes at 28 and 188 dpn. In contrast, in liver at a late prenatal stage a steady state was observed, and at later stages a significant modulation and different responsiveness of the transcriptome was observed [9]. Overall the study indicates that pigs show a high level of developmental plasticity that allow adaptation to a high protein:low carbohydrate maternal environment possibly related to metabolic energy deficiency *in utero* on the organismal level, paralleled by an enduring modulation of the transcriptome. However, the altered mRNA abundance of genes related to energy metabolism may indicate a higher susceptibility of offspring to metabolic challenges, leading to predisposition for metabolic disturbances at later adulthood when offspring is exposed to physiological or nutritional challenging situations.

Materials and Methods

Animals and sample collection

Animal care and tissue collection were performed according to guidelines of the German Law of Animal Protection and with approval by the Animal Care Committee of the State Mecklenburg-Vorpommern (Landesamt fr Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LVL MV/TSD/7221.3-1.1-006/04; LALLF M-V/TSD/7221.3-1.2-05/06; LALLF M-V/TSD/7221.3-1.2-013/06). Experimental diets were administered as described [2]. Briefly, at insemination German Landrace primiparous sows (n = 42) were randomly assigned to either a high-protein diet (HP) with 30% (w/w) crude protein or an adequate-protein diet (AP) containing 12% crude protein. Diets were formulated to be isoenergetic ($\approx 13.7 MJME/kg$) by adjustment of the carbohydrate component of the diet (HP; 30% crude protein; protein: carbohydrate ratio 1:1.3; AP; 12% crude protein; protein: carbohydrate ratio 1:5) [2]. Tissue sampling included offspring of these sows at one prenatal [94 days post conception (dpc)] and three postnatal [1,28, 188 days post natum (dpn)] time points (Figure 3).

At 94 dpc, a subset of three sows per dietary group was subjected to Caesarean section. Eight viable fetuses per sow were collected starting at the tip of the left uterine horn and alternating between left and right horn. Fetuses were killed by i.v. injection of T61 in the *V.cava cranialis* and muscle samples (*Musculus longissimus dorsi*) were immediately collected (approximately 500 mg), frozen in liquid nitrogen, and stored at -80°C until analysis. All litters sampled comprised at least 11 viable piglets. There were no differences in the number of fetuses per litter between the gestation diet groups. Fetuses of HP fed dams had a lower mean weight compared to AP fetuses at 94 dpc (HP: 608 ± 107 g, and AP: 701 ± 103 g, respectively; $P = 0.16$; $n = 12$). The lightest and the heaviest fetuses within one litter were selected for transcriptome analysis.

Offspring collected for postnatal time points were born to primiparous sows after prostaglandin induction of parturition as described [2] and were farrowed after mean pregnancy duration of 115 days. Offspring of six sows per diet with a minimum of 11 liveborn piglets (median litter size = 13) were used; litter size was not different among groups. At birth 10 piglets in each litter were distributed to groups for three time points (1, 28, and 188 dpn). For microarray analyses, six sex-balanced sib pairs (all stages) were chosen per stage and diet. At 1 dpn the lightest and the heaviest piglets within one litter were selected; at later stages body weight was not a criterion for selection but was recorded for consideration in the statistical evaluation. Considering all piglets derived from the complete experiment, mean birth weights of HP newborn piglets were lower than birth weights of AP offspring (HP: 1.21 ± 0.04 kg, and AP: 1.41 ± 0.04 kg respectively, $P \leq 0.05$) [2,13]. However, in the smaller subset of samples used for microarray analyses, differences in body weights between HP and AP offspring did not reach statistical significance (HP: 1.27 ± 0.32 kg, and AP: 1.28 ± 0.43 kg, respectively; $P = 0.96$; $n = 12$). Thirty-six hours after birth, the lightest and the heaviest piglet within one litter were killed by i.m. injection of 1.25 mg

propionyl-promazine (0.2 ml Combelen, Bayer AG, Leverkusen, Germany) and 50 mg ketamine (Ursotamin, Serumwerk Bernburg AG, Germany). Samples were immediately collected from *M. longissimus dorsi* (approximately 500 mg), frozen in liquid nitrogen, and stored at -80°C until analysis.

Remaining piglets were cross-fostered to multiparous sows, which were fed AP diets during gestation and lactation. Litter size during suckling was standardized to 11 piglets per sow. Male piglets were castrated at 4 dpn. From weaning (28 dpn) to slaughter (188 dpn), all piglets were individually reared. They had free access to standard diets formulated for postweaning (29 to 76 dpn), growing (77 to 105 dpn), and finishing periods [13,14]. At 28 dpn (HP: 7.98 ± 1.51 kg, and AP: $7.78 \pm \pm 2.31$ kg respectively; $P = 0.86$; $n = 12$) and 188 dpn of age (HP: 130.57 ± 8.01 kg, and AP: 132.47 ± 19.58 kg respectively; $P = 0.83$; $n = 12$), pigs were weighed after an overnight fast and killed by electronarcosis followed by exsanguination in the experimental slaughterhouse of FBN. Muscle tissue was immediately collected from M. longissimus dorsi, frozen in liquid nitrogen, and stored at -80°C until use for RNA isolation.

RNA isolation, target preparation, and hybridization

Total RNA from individual muscle samples was isolated using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany) and subsequently subjected to DNase treatment and a column-based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity and quantity were checked by agarose gel electrophoresis and by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). Absence of a DNA contamination was verified by PCR of the porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Forward primer: AAGCAGGGATGATGTTCTGG; Reverse primer: ATGCCTCCTGTACCACCAAC) with isolated RNA as template. All RNA samples were stored at -80°C until downstream analysis. For the microarray experiments individual biotin-labeled cDNA was synthesized by the Gene Chip 3′ Express Kit (Affymetrix, Santa Clara, CA, USA). cDNA was fragmented ($\approx 100bp$) and hybridized on Affymetrix GeneChip Porcine Genome Arrays. After staining and washing steps the arrays were scanned (Affymetrix, Santa Clara, CA, USA).

Data analysis

Bioinformatic analysis was done in R [47]. First, a quality control was performed. Except for the AP group at 188 dpn (4 samples), all diet group and stage combinations had 6 samples meet the appropriate quality control criteria. Samples were GC-RMA normalized (Log2) over all stages. The MAS5 algorithm was used to skip those transcripts expressed in less than 50% of the animals within one dietary group per stage. For a second filtering step standard deviations were calculated for each probe set over all subsets of arrays of the particular comparisons. Probe sets with a low standard deviation ($s \leq 0.25$) were discarded because such transcripts are not likely to show an altered abundance. Relative changes in mRNA levels were determined using a mixed model analysis, including effects of dietary treatment, stage, sex, weight (as deviation from the mean weight within stage, in percent), and interaction between diet and stage, as well as mother as a random effect. P-values (significance set at $P \leq 0.05$) for each comparison were converted to a set of q-values (≤ 0.25) using the algorithm proposed by Storey and Tibshirani [48].

Throughout the manuscript, results are given for the comparisons in the direction of HP vs. AP; thus 'increased abundance' indicates higher transcript abundance in HP than in AP. Analysis of the pathways involved was carried out using Ingenuity Pathway Analysis [49]. The up-to-date annotation of Affymetrix probe sets to EnsEMBL Sscofa 9 (20,439 of 23,935 annotated probe sets) was used [15]. All the microarray data are MIAME compliant, and the raw data have been deposited in a MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) (accession numbers: GSE33737, GSE33738).

Pathway analysis

Gene lists from microarray results were submitted to Ingenuity Pathways Analysis (Ingenuity) to assign the altered genes to biofunctions and canonical pathways. The focus was on those canonical pathways appearing at least once within the top ten altered pathways within one single analysis. Interactions presented in the networks are not specific for porcine muscle tissue, as the database contains literature from many different research areas.

Author Contributions

Conceived and designed the experiments: KW CCM. Performed the experiments: MO SP. Analyzed the data: KW MO SP EM. Contributed reagents/materials/analysis tools: KW EM SP CCM. Wrote the paper: MO KW.

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2.5 Transcriptional response of skeletal muscle to low protein gestation diet in porcine progeny accumulates in growth- and cell cycle-regulating pathways

Michael Oster, Eduard Murani, Cornelia C. Metges, Siriluck Ponsuksili and Klaus Wimmers

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Transcriptional response of skeletal muscle to a low-protein gestation diet in porcine offspring accumulates in growth- and cell cycle-regulating pathways

Michael Oster,[1] Eduard Murani,[1] Cornelia C. Metges,[2] Siriluck Ponsuksili,[3] and Klaus Wimmers[1]

[1]*Research Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany;*
[2]*Research Unit Nutritional Physiology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany;*
and [3]*Research Group Functional Genomics, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany*

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Oster M, Murani E, Metges CC, Ponsuksili S, Wimmers K. Transcriptional response of skeletal muscle to a low-protein gestation diet in porcine offspring accumulates in growth- and cell cycle-regulating pathways. *Physiol Genomics* 44: 811–818, 2012. First published July 3, 2012; doi:10.1152/physiolgenomics.00050.2012.—Inadequate maternal protein supply during gestation represents an environmental factor that affects physiological signaling pathways with long-term consequences for growth, function, and structure of various tissues. Hypothesizing that the offspring's transcriptome is persistently altered by maternal diets, we used a porcine model to monitor the longitudinal expression changes in muscle to identify pathways relevant to fetal initiation of postnatal growth and development. German Landrace gilts were fed isoenergetic gestational diets containing 6.5% (LP) or 12.1% protein. The longissimus dorsi samples were collected from offspring at 94 days postconception (dpc) and 1, 28, and 188 days postnatum (dpn) for expression profiling. At 94 dpc, 1 dpn, and 28 dpn relatively few transcripts (<130) showed an altered abundance between the dietary groups. In fact, at 94 dpc genes of G2/M checkpoint regulation and mitotic roles of Polo-like kinases showed lowered transcript abundance in LP. At 188 dpn 677 transcripts were altered including those related to oxidative phosphorylation, citrate cycle, fatty acid metabolism (higher abundance in LP) and cell cycle regulation (lower abundance in LP). Correspondingly, transcriptional alterations during pre and postnatal development differed considerably among dietary groups, particularly for genes related to cell cycle regulation (G1/S and G2/M checkpoint regulation; cyclines), growth factor signaling (GH, IGF1, mTOR, RAN, VEGF, INSR), lipid metabolism, energy metabolism, and nucleic acid metabolism. In skeletal muscle, fetal programming related to maternal LP diets disturbed gene expression in growth-related pathways into adulthood. Diet-dependent gene expression may hamper proper development, thereby affecting signaling pathways related to energy utilization.

fetal programming; microarray; intrauterine growth restriction

IN MURINE AND PORCINE MODELS a relationship has been demonstrated between maternal protein supply during pregnancy and birth weight (33, 39), body composition (5), and metabolic disorders (9, 19, 31) of offspring. Furthermore, analysis of porcine skeletal muscle tissue revealed prenatal effects on the postnatal phenotype, including myofiber number and size as well as restricted muscle growth (34). Collectively, these observations are in accordance with the "thrifty phenotype hypothesis" (13) that proposes the relationship between low birth weight, i.e., intrauterine growth restriction (IUGR), and a higher propensity for adult chronic metabolic diseases. The possible molecular mechanism explaining the intrauterine adaptive response to adverse environmental changes is termed "fetal programming." Increasing evidence indicates that dietary protein intake below requirement throughout gestation is associated with alterations in gene expression in a species-, tissue-, and stage-specific manner (12, 21, 28, 42). To gain knowledge about transcriptional mechanisms underlying the adaptive response to inadequate nutritional supply, we applied whole-genome microarrays for expression profiling in a longitudinal experimental design. Pregnant German Landrace gilts were fed isoenergetic gestational diets containing either adequate protein (AP) or low protein (LP) at the expense of carbohydrate supply. The offspring were sampled at prenatal and postnatal time points (33). In our experiment, offspring exposed to a maternal dietary undersupply of protein and appropriate postnatal dietary conditions exhibited "catch-up" growth through 28 days (34).

In fact, as previously reported, newborns from sows that received a LP supply during gestation had a significantly lower birth weight, a lower body fat content, and reduced size and number of adipocytes and muscle fibers than newborns of the control group (34). At weaning [28 days postnatum (dpn)] offspring of the LP group had significantly (albeit slightly) higher fat content and adipocyte size but still lower muscle fiber numbers. However, neither body weight at weaning nor body weight at 188 dpn differed significantly between offspring of the LP and the AP group, whereas visceral and subcutaneous fat content remained higher in LP than in AP during postnatal life (33–35). We have previously demonstrated that, depending on the gestational diet, the expression profile of the liver, the central metabolic organ, was affected at both prenatal and postnatal stages; specifically, an altered hepatic expression of genes related to cell cycle and cell maintenance as well as lipid, ketone body, and amino acid metabolism was observed (28). Here, we focus on muscle tissue, representing the largest peripheral consumer of energy and nutrients, contributing to the species-typical shape of the body, and being a main agricultural product for human consumption. We show that the transcript abundances in skeletal muscle were modulated during prenatal and postnatal stages, i.e., an acute and delayed response to the nutritional stimulus is obvious.

MATERIALS AND METHODS

Animals and sample collection. Animal care and tissue collection were performed according to guidelines of the German Law of Animal Protection and with approval by the Animal Care Committee of the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LVL MV/TSD/7221.3-1.1-006/04; LALLF MV/TSD/7221.3-1.2-05/06; LALLF M-V/TSD/7221.3-1.2-013/06). Experimental diets were administered as described (33). In brief, at insemination German Landrace primiparous sows ($n = 42$) were randomly assigned to either an LP diet with 6.5% (wt/wt) crude protein or an AP diet containing 12.1% crude protein. Diets were formulated to be isoenergetic (~13.7 MJ ME/kg) by

Address for reprint requests and other correspondence: K. Wimmers, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany (e-mail: wimmers@fbn-dummerstorf.de).

adjustment of the carbohydrate component of the diet (LP: 6.5% crude protein, protein-carbohydrate ratio 1:10; AP: 12.1% crude protein, protein-carbohydrate ratio 1:5) (33). Tissue samples ($n = 6$ per stage and dietary group) were collected from offspring of these sows at one prenatal [94 days postconception (dpc)] and three postnatal (1, 28, 188 dpn) time points (Fig. 1).

At 94 dpc, a representative subset of three sows per dietary group was subjected to Caesarean section. Eight viable fetuses per sow were collected starting at the tip of the left uterine horn and alternating between left and right horn. Fetuses were killed by iv injection of T61 in the V. cava cranialis and muscle samples (Musculus longissimus dorsi) were immediately collected (~500 mg), frozen in liquid nitrogen, and stored at −80°C until analysis. All litters sampled had at least 11 viable piglets. There were no differences in the number of fetuses per litter between the gestation diet groups (24). However, fetuses from LP-fed dams had numerically lower weight compared with AP fetuses at 94 dpc (lsm ± SE, LP: 635.9 ± 18.8 g; AP: 687.4 ± 18.8 g; $P \geq 0.05$) (24).

Offspring collected for postnatal time points were born to primiparous sows after prostaglandin induction of parturition as described (33) and was born after a mean pregnancy duration of 115 days. Offspring of six sows per diet with a minimum of 11 live born piglets (median litter size = 13) were used; litter size was not different among groups. At birth the piglets of each litter were allocated to groups at three time points (1, 28, and 188 dpn). Mean birth weights of LP newborn piglets were lower than birth weights of AP offspring (lsm ± SE, LP: 1.19 ± 0.04 kg; AP: 1.41 ± 0.04 kg, $P \leq 0.05$) (33). Thirty-six hours after birth, the lightest and the heaviest piglets within one litter were killed by injection of 1.25 mg propionyl-promazine im (0.2 ml Combelen; Bayer, Leverkusen, Germany) and 50 mg ketamine (Ursotamin; Serumwerk Bernburg, Bernburg, Germany). Samples of M. longissimus dorsi (~500 mg) were immediately collected, frozen in liquid nitrogen, and stored at −80°C until analysis.

Remaining piglets were cross-fostered to multiparous sows, which were fed AP diets during gestation and lactation. Litter size during suckling was standardized to 11 piglets per sow. Male piglets were castrated at 4 dpn. From weaning (28 dpn) to slaughter (188 dpn), all piglets were individually reared. They had free access to standard diets formulated for postweaning (29–76 dpn), growing (77–105 dpn), and finishing periods (33). At 28 dpn and 188 dpn pigs were weighed after an overnight fast and killed by electronarcosis followed by exsanguination in the experimental slaughterhouse of FBN. At 28 dpn (lsm ± SE, LP: 7.60 ± 0.34 kg; AP: 7.70 ± 0.34 kg; $P \geq 0.05$) and 188 dpn (lsm ± SE, LP: 125.60 ± 2.40 kg; AP: 131.30 ± 2.49 kg; $P \geq 0.05$) the diet groups did not differ in body weight (34, 35). Muscle tissue was immediately collected from M. longissimus dorsi, frozen in liquid nitrogen, and stored at −80°C until use for RNA isolation.

For microarray analyses, six sib pairs were chosen from each stage and diet to equally represent the experimental groups in terms of gender. At 94 dpc and 1 dpn the lightest and the heaviest piglets within one litter were selected (with three male and three female offspring belonging to either the light or the heavy group); at later stages body weight was not a criterion for selection but was recorded for consideration in the statistical evaluation.

RNA isolation, target preparation, and hybridization. Total RNA from individual muscle samples was isolated using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany) and subsequently subjected to DNase treatment and a column-based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity and quantity were checked by agarose gel electrophoresis and by spectrometry with a NanoDrop ND1000 spectrophotometer (PEQLAB, Erlangen, Germany). Absence of DNA contamination was verified by PCR of the porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward primer, AAGCAGGGATGATGTTCTGG; reverse primer, ATGCCTCCTGTACCACCAAC) with isolated RNA as template. All RNA samples were stored at −80°C until downstream analysis. For the microarray experiments individual biotin-labeled cDNA was synthesized by the Gene Chip 3′ Express Kit (Affymetrix, Santa Clara, CA). cDNA was fragmented (~100 bp) and hybridized on Affymetrix GeneChip Porcine Genome Arrays. After staining and washing steps the arrays were scanned (Affymetrix).

Data analysis. Bioinformatic analysis was done in R (36). First, a quality control was performed. Except for the AP group at stage 188 dpn (4 samples) and the LP group at stage 94 dpn (5 samples), all diet group and stage combinations had six samples that passed the appropriate quality control criteria. Samples were GC-RMA normalized (Log2) over all stages. The MAS5 algorithm was used to skip those transcripts that were expressed in <50% of the animals within one dietary group per stage. For a second filtering step SDs were calculated for each probe set over all subsets of arrays of the particular comparisons. Probe sets with a low SD (≤ 0.25) were discarded, because such transcripts are not likely to show an altered abundance. Relative changes in mRNA levels were determined by a mixed-model analysis, including effects of dietary treatment, stage, sex, weight (as variation of the mean weight within stage in percent), and sow as a random effect confounded with dietary treatment. The interaction between dietary treatment and ontogenetic stage refers to the longitudinal experimental design. P values (significance set at $P \leq 0.05$) for each comparison were converted to a set of q values ($q \leq 0.25$) using the algorithm proposed by Storey and Tibshirani (40).

Throughout this article, results are given for the comparisons in the direction of LP vs. AP; thus, "increased abundance" indicates higher transcript abundance in LP than in AP. Analysis of the pathways involved was carried out using Ingenuity Pathway Analysis (IPA) (15). The up-to-date annotation of Affymetrix probe sets to EnsEMBL Sscofa 9 (20,439 of 23,935 annotated probe sets) was used (27). All the microarray data are MIAME compliant, and the raw data have been deposited in an MIAME compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) (accession numbers: GSE33737 and GSE33739).

Pathway analysis based on IPA. Gene lists from microarray results were submitted to IPA to assign the altered genes to biofunctions and canonical pathways. With regard to the IPA features, canonical

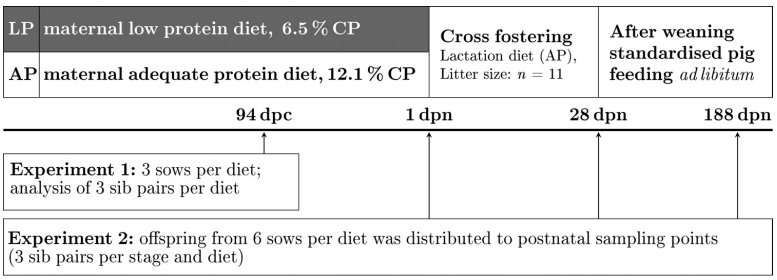

Fig. 1. Experimental design. Fetuses and offspring of differently fed sows were collected at 4 developmental stages. Fetuses were derived from 3 sows per dietary group. Postnatal offspring were full sibs of 6 litters per dietary group collected at 3 consecutive postnatal stages. LP, low protein; CP, crude protein; AP, adequate protein.

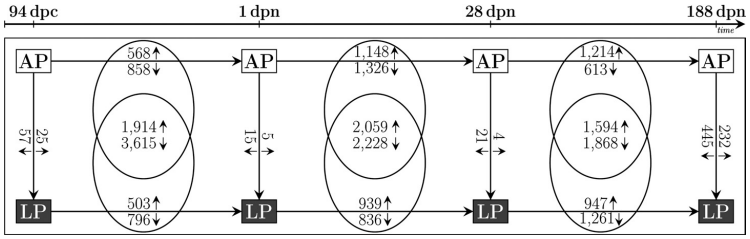

Fig. 2. Number of probe sets showing a significantly altered abundance in muscle tissue. The number of altered probe sets between adjacent developmental stages in AP or LP offspring are indicated at horizontal arrows; the number of commonly altered probe sets between stages in AP and LP offspring are indicated at intersections; the number of probe sets showing a different abundance between LP and AP offspring at the same developmental stage are indicated at vertical arrows; small arrows at the numbers indicate a higher or lower probe set abundance, respectively.

pathways were seen as IPA pathways. The focus was on those IPA pathways appearing at least once within the top-10 altered pathways within one single analysis. Interactions presented in the networks are not specific for porcine muscle tissue, as the database contains literature from many different research areas.

RESULTS

Diet-dependent effects associated with mechanisms of fetal programming were investigated in muscle tissue, covering one prenatal and three postnatal stages. Expression patterns of LP and AP animals were evaluated at each developmental stage (Figs. 2 and 3, vertical arrows). Furthermore, transcriptional shifts between adjacent stages among the dietary groups (Figs. 2 and 3, horizontal arrows) were investigated. Thus, information was acquired regarding diet-dependent adaptation processes during development.

In comparing LP vs. AP, we found 15,526 probe sets to be expressed according to MAS5 analysis in muscle tissue. Further filtering based on the variability of expression of probe sets revealed 14,058 probe sets for further analysis. These probe sets represent 10,028 genes according to the recent annotation (27).

Comparisons between LP and AP within stages. The mRNA expression patterns in LP and AP offspring was compared within each developmental stage (Fig. 2). At the prenatal stage (94 dpc) 82 probe sets were found to differ significantly between LP and AP fetuses (25 LP > AP). Specifically, mRNA transcript levels of genes associated with G2/M DNA damage checkpoint regulation and mitotic roles of Polo-like kinases were decreased in LP fetuses compared with AP fetuses (Table 1, Fig. 3). In neonatal piglets (1 dpn) 20 probe sets differed between the experimental groups (5 LP > AP). In juvenile piglets at weaning (28 dpn) 25 probe sets differed significantly between LP and AP offspring (4 LP > AP). Due to the marginal differences in mRNA abundance at 1 dpn and 28 dpn, no distinct metabolic pathway was found to exhibit diet-dependent alterations in neonatal and juvenile piglets. In adult pigs (188 dpn) 677 probe sets were significantly different between LP and AP offspring. The abundance of 232 probe sets was increased in the LP offspring compared with AP offspring. Genes associated with oxidative phosphorylation, citrate cycle, and fatty acid metabolism showed increased mRNA expression levels, whereas genes associated with G1/S checkpoint regulation as well as cyclin and cell cycle regulation decreased expression in LP offspring.

Differences in longitudinal developmental changes of transcript abundance among LP and AP offspring. Expression patterns of two adjacent developmental stages within each dietary group were compared (Fig. 1) to determine transcripts showing an altered abundance between stages ($P \leq 0.05$) (Fig. 2). Resulting gene lists were likened between LP and AP offspring at the corresponding developmental periods. The intersection of commonly altered genes (Supplemental Table S1) between those comparisons represents genes that showed the same shift of abundance along stages in both dietary groups and, thus, can be assumed to reflect normal physiological maturation.[1] The analysis focused on those transcripts whose change of abundance between two consecutive stages (*period 1*: 94 dpc–1 dpn; *period 2*: 1–28 dpn; *period 3*: 28–188 dpn)

[1] The online version of this article contains supplemental material.

Table 1. *Functional annotation of muscle transcripts showing altered abundance depending on the dietary group (LP vs. AP) within different developmental stages (IPA)*

Developmental Comparison	Affected IPA Pathway	Expression	*P* Value	No. of Genes Involved, *n*	Genes Involved in Pathway
94 dpc	G2/M DNA damage checkpoint regulation	down	6.10·E-3	2	CCNB2, CHEK1
	mitotic roles of Polo-like kinase	down	1.11·E-2	2	CCNB2, KIF11
1 dpn					
28 dpn					
188 dpn	oxidative phosphorylation	up	4.10·E-3	6	CYC1, NDUFA3, NDUFV2, SDHA, UQCR11, UQCRC1
	citrate cycle	up	3.32·E-3	8	DLST, IDH3G, SDHA
	fatty acid metabolism	up	6.23·E-5	8	ACAD9, ACADS, ACADVL, CPT1A, CPT1B, CYP2D6, DCI, HADHA
	cyclin and cell cycle regulation	down	3.68·E-3	6	ATR, CDK6, E2F3, E2F5, PPP2CB, PPP2R5E
	G1/S checkpoint regulation	down	3.95·E-3	5	ATR, CDK6, E2F3, E2F5, SMAD4

"Up" and "down" indicate higher and lower abundance in low protein (LP) compared with adequate protein (AP) groups, respectively. IPA, Ingenuity Pathway Analysis. dpc, days postconception; dpn, days postnatum. *P* value, significance of association between dataset and IPA pathways; Fischer's exact test.

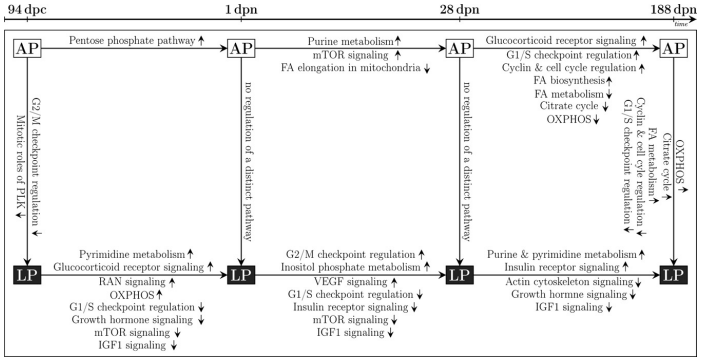

Fig. 3. Affected pathways in muscle tissue between developmental stages and diets. Listed pathways between AP stages (white boxes) indicate shifts during development that are not found in LP offspring (black boxes) at the corresponding period. Pathways between the LP stages indicate alterations that occur in LP offspring but not in AP offspring in the corresponding period. Arrows between boxes show direction of comparison; small arrows indicate higher and lower transcript abundance, respectively. OXPHOS, oxidative phosphorylation; PLK, Polo-like kinase; mTOR, mammalian target of rapamycin; FA, fatty acid; IGF1, insulin-like growth factor 1; RAN, Ras-related nuclear protein; VEGF, vascular endothelial growth factor.

belonged to the symmetric difference in either the LP or the AP group. Thus, genes and pathways identified as altered in one experimental group displayed diet-dependent longitudinal transcriptomic alterations.

When fetuses and perinatal offspring were compared (developmental *period 1*) 1,426 probe sets (568 94 dpc $<$ 1 dpn in AP) differed in their frequency and direction of change between the AP group and the LP group. Genes associated with pentose phosphate pathway had increased expression at 1 dpn (Table 2). In LP offspring 1,299 probe sets showed diet-specific differences in their abundance (503 94 dpc $<$ 1 dpn in LP) during the corresponding time period compared with the AP group. The mRNA abundance of genes associated with pyrimidine metabolism, glucocorticoid receptor signaling, RAN signaling, and oxidative phosphorylation was higher in LP perinatal piglets. Furthermore, genes participating in G1/S checkpoint regulation, growth hormone signaling, mammalian target of rapamycin (mTOR) signaling, and IGF1 signaling exhibited decreased expression in LP perinatal piglets.

Comparing perinatal and juvenile piglets (*period 2*), we found 2,474 probe sets were altered in AP offspring compared with LP offspring. Of these, 1,148 probe sets showed a higher abundance and 1,326 probe sets were detected at lower abundances at a higher age in AP but not in LP. Expression values of genes participating in purine metabolism and mTOR signaling were increased, while genes associated with fatty acid elongation in mitochondria were decreased in AP juvenile offspring. In the same period, differences in mRNA expression represented by 1,775 probe sets were specific to LP offspring. Of these, 939 probe sets had an increased mRNA abundance and represented genes involved in G2/M DNA damage checkpoint regulation, inositol phosphate metabolism, and VEGF signaling. Furthermore, genes associated with G1/S checkpoint regulation, insulin receptor signaling, mTOR signaling, and IGF1 signaling had lower transcript abundance in LP offspring at 28 dpn.

When juvenile and young adult pigs (*period 3*) were compared 1,827 probe sets differed significantly (1,214 28 dpn $<$ 188 dpn in AP) in AP offspring. Young adult AP offspring had higher expression of transcripts participating in G1/S checkpoint regulation, glucocorticoid receptor signaling, and fatty acid biosynthesis, as well as cyclin and cell cycle regulation. Expression was lowered in transcripts associated with fatty acid metabolism, citrate cycle, and oxidative phosphorylation in adult AP pigs. In LP offspring 2,208 probe sets were differentially expressed (947 28 dpn $<$ 188 dpn in LP). Genes involved in insulin receptor signaling as well as purine and pyrimidine metabolism displayed higher abundance in adult pigs, while transcripts involved in actin cytoskeleton signaling, growth hormone signaling and IGF1 signaling had lowered expression.

A comprehensive overview of the pathways altered between diets (LP vs. AP) and stages (94 dpc; 1, 28, 188 dpn) is depicted in Fig. 3.

DISCUSSION

Stage specificity of the transcriptional response to LP maternal gestation diet. Whole-genome microarrays were used to analyze muscle gene expression profiles of offspring exposed to maternal isoenergetic LP or AP diets throughout fetal development. This longitudinal experiment comprised prenatal, perinatal, juvenile, and adult developmental stages in a porcine model. We sought to analyze diet- and stage-dependent gene expression as affected by maternal protein sufficiency. Because offspring were raised in a controlled and identical nutritional management by cross-fostering and whole body changes in tissue composition at adult stage were subtle (35), we interpret observed changes in 188 dpn as being a result of fetal programming enabling postnatal adaptation processes.

The direct comparisons of mRNA abundances at prenatal, perinatal, and juvenile stages revealed only slight differences among dietary groups. In terms of the number of transcripts with an altered abundance, the peripheral tissue of skeletal muscle showed low acute response to inadequate nutritional supply. In particular, at 94 dpc, 1 dpn, and 28 dpn, expression patterns of skeletal muscle tissue revealed only subtle differences. However, the resulting transcriptional phenotype at 188 dpn showed considerable divergence between the LP and AP groups. Our longitudinal holistic study design is able to trace stage-dependent modulations of the offspring's transcriptome along development, revealing even more distinct differences between dietary groups. Only later in life, at 188 dpn, do shifts of developmental changes of transcript abundance accumulate in a considerable long-term transcriptional response to the prenatal intervention that can be regarded as fetal nutritional programming. Regarding species-specific maturation differences, the number of transcripts differing in their mRNA

Table 2. *Functional annotation of muscle transcripts showing altered abundance between two developmental stages within either dietary group LP or AP (IPA)*

Developmental Comparison	Diet	Affected IPA Pathway	Expression	*P* Value	Genes Involved, *n*	Genes Involved in Pathway
94 dpc vs. 1 dpn	AP	pentose phosphate pathway	up	1.35·E-2	4	ALDOA, DERA, FBP2, PDHB
	LP	pyrimidine metabolism	up	4.45·E-2	7	CTPS2, ENTPD4, POLR2C, POLR2G, POLR2L, RRM2B, UCK1
	LP	glucocorticoid receptor signaling	up	4.24·E-2	10	CD163, ESR1, GTF2A1, HSPA2, NCOR2, NFATC3, POLR2C, POLR2G, POLR2L, STAT3
	LP	RAN signaling	up	4.32·E-2	2	KPNA1, KPNA3
	LP	oxidative phosphorylation	up	3.84·E-2	7	ATP6V0B, ATP6V0D1, ATP6V1F, COX6B1, NDUFB9, NDUFS3, TNNI2
	LP	G1/S checkpoint regulation	down	4.16·E-2	5	ATR, CDK4, HDAC6, SMAD4, TGFB1
	LP	growth hormone signaling	down	8.30·E-3	7	GHR, IGF1, IGFBP3, IRS1, PIK3R2, PRKCH, RPS6KA5
	LP	mTOR signaling	down	2.69·E-2	10	AKT3, EIF4B, IRS1, PIK3R2, PLD3, PPP2CB, PPP2R5E, PRKCH, RPS6KA5, VEGFC
	LP	IGF1 signaling	down	1.32·E-2	8	AKT3, IGF1, IGFBP3, IGFBP5, IRS1, PIK3R2, PRKACB, YWHAB
1 dpn vs. 28 dpn	AP	purine metabolism	up	3.51·E-3	26	ACIN1, ATIC, ATP11B, ATP5G2, ATP6V0B, BCKDHA, CHRAC1, DDX19B, DGUOK, DLG1, GUCY1B3, NME3, PDE2A, PDE5A, POLB, POLE2, POLG, POLR2D, POLR2F, POLR2J, POLR3K, PRPSAP2, PSMC5, RSF1, TRAP1, VPS4B
	AP	mTOR signaling	up	8.73·E-3	14	AKT1S1, EIF3B, EIF3G, EIF3I, EIF4EBP1, EIF4G1, FNBP1, HIF1A, MAPKAP1, NAPEPLD, PRKAG1, RHOC, RHOJ, RRAS
	AP	fatty acid elongation in mitochondria	down	9.88·E-3	4	ACAA2, HSD17B4, MECR, PECR
	LP	G2/M DNA damage checkpoint regulation	up	3.72·E-2	5	CCNB1, RPRM, SKP2, YWHAE, YWHAG
	LP	inositol phosphate metabolism	up	3.24·E-2	11	CDK6, IMPA1, INPP1, IP6K2, MAP2K4, PDIA3, PIK3CG, PIP4K2C, PLCB3, PLCD1, PRKCH
	LP	VEGF signaling	up	1.38·E-2	9	ACTN1, EIF2S2, EIF2S3, PIK3CG, SOS1, SOS2, VEGFA, VEGFC, YWHAE
	LP	G1/S checkpoint regulation	down	3.00·E-3	7	CDK2, HDAC3, MYC, RBL2, SMAD3, TGFB3, TP53
	LP	insulin receptor signaling	down	6.00·E-4	13	AKT2, EIF2B5, EIF4EBP1, FOXO1, GRB10, LIPE, PIK3R1, PPP1CA, PPP1R14B, PRKAG1, SCNN1G, SHC1, SLC2A4
	LP	mTOR signaling	down	1.54·E-3	13	AKT2, EIF3D, EIF3F, EIF4A3, EIF4EBP1, PIK3R1, PLD3, PPP2R1A, PRKAG1, PRKD3, RHOB, RHOC, RPS6KA3
	LP	IGF1 signaling	down	4.44·E-3	9	AKT2, CTGF, FOS, FOXO1, GRB10, JUN, PIK3R1, PRKAG1, SHC1
28 dpn vs. 188 dpn	AP	G1/S checkpoint regulation	up	5.54·E-3	8	ATR, CDKN1B, E2F5, GSK3B, RB1, RBL1, RBL2, TGFB2
	AP	glucocorticoid receptor signaling	up	1.36·E-2	21	CREBBP, ESR1, FKBP5, GTF2B, GTF2H1, HSP90AA1, HSPA2, HSPA4, JAK2, MAPK8, MED1, PIK3C3, PPP3CA, PRKACB, SERPINE1, SMARCA2, SOS2, TAF4, TAF9, TAF13, TGFB2
	AP	cyclin and cell cycle regulation	up	1.46·E-2	9	ATR, CDKN1B, E2F5, GSK3B, PPP2CB, PPP2R2A, PPP2R5E, RB1, TGFB2
	AP	fatty acid biosynthesis	up	1.83·E-2	3	ACSL3, BTD, MCCC2
	AP	fatty acid metabolism	down	8.72·E-3	9	ACAD9, ACADL, ACADS, ACADVL, CPT1A, HADHB, HSD17B10, IVD, PECI
	AP	citrate cycle	down	1.08·E-4	6	ACO2, FH, IDH2, IDH3G, SDHA, SDHB
	AP	oxidative phosphorylation	down	4.00·E-5	14	ATP5L, ATP6AP1, ATP6V0C, COX10, NDUFA2, NDUFA3, NDUFB7, NDUFS2, NDUFS3, NDUFS8, NDUFV2, SDHA, SDHB, TNNI2

Continued

Table 2.—*Continued*

Developmental Comparison	Diet	Affected IPA Pathway	Expression	*P* Value	Genes Involved, *n*	Genes Involved in Pathway
	LP	purine metabolism	up	1.79·E-3	23	ABCD3, ATIC, ATP13A2, BCKDHA, DDX1, ENTPD7, ERCC3, GMPR, MGC13098, MPP1, POLG, POLR1A, POLR2C, POLR2D, POLR2F, POLR3D, PRPS1, PSMC1, PSMC2, PSMC5, RRM2B, VCP, VPS4B
	LP	pyrimidine metabolism	up	2.32·E-3	14	ENTPD7, MGC13098, NXN, POLG, POLR1A, POLR2C, POLR2D, POLR2F, POLR3D, RPUSD4, RRM2B, TRUB2, UCK1, UPB1
	LP	insulin receptor signaling	up	4.48·E-4	14	EIF2B1, EIF2B2, EIF2B5, FOXO1, GRB10, INPPL1, IRS1, PPP1CB, PPP1CC, PRKAR2A, RAC3, RHOQ, SLC2A4, TSC2
	LP	actin cytoskeleton signaling	down	4.97·E-3	21	ABI2, ACTG2, ACTN1, ACTN2, ACTR3, ARHGEF6, ARPC3, CFL2, CYFIP1, EZR, GNG12, GRB2, ITGB1, KRAS, MAP2K1, MYH10, MYL4, PIK3CG, PIP4K2A, ROCK1, VAV2
	LP	growth hormone signaling	down	9.81·E-3	9	IGF1, IGF1R, IGFBP3, PDPK1, PIK3CG, PRKD3, RPS6KA5, SRF, STAT1
	LP	IGF1 signaling	down	3.50·E-3	12	GRB2, IGF1, IGF1R, IGFBP2, IGFBP3, KRAS, MAP2K1, PDPK1, PIK3CG, PRKAR1A, PRKAR2B, SRF

"Up" and "down" indicate higher and lower abundance in later compared with earlier stages, respectively. *P* value, significance of association between dataset and IPA pathways; Fischer's exact test.

abundances between the dietary groups is consistent with expression analyses done in rodents, in which dietary modifications during pregnancy were investigated. Investigating adult animals (84 dpn–450 dpn), the analyses revealed diet-dependent differences in mRNA expression in liver (21, 26), muscle (30), kidney (1), pancreas (41), and adipose tissue (2, 12). In this context, a great variety of metabolic processes were described, including mitochondrial biogenesis, cell differentiation, lipid metabolism, and genes associated to hypertension. Hence, transcript abundance associated with fetal programming is highly specific to both ontogenetic stage and analyzed tissue.

Molecular pathways with affected transcript abundance. Expression analysis revealed a number of pathways associated to growth, cell cycle regulation, carbohydrate metabolism, lipid metabolism, and stress response that were altered at various stages in skeletal muscle tissue due to a maternal LP diet. In our experiment, LP diets fed to sows during pregnancy led to growth restriction of offspring at the neonatal stage. Newborns from sows that received a LP supply during gestation had significantly lower body weight, associated with lower body fat content and reduced adipocyte and muscle fiber number and size, than newborns of the control group (33, 34). Consistently, the massive shift toward a lowered transcriptional abundance of genes in growth-related pathways within fetuses (94 dpc) and developmental *period 1* in LP offspring pointed to diet-dependent transcriptional processes that may contribute to IUGR. The observed transcriptional shifts indicate disturbances in muscle growth, which is in line with observations of adversely-affected myogenesis, delayed muscle maturation, and less differentiation at birth in response to the LP diet (34). Interestingly, the offspring that were exposed to an undersupply of protein during fetal development but had appropriate postnatal dietary conditions were able to broadly adapt in terms of body weight (34). However, transcriptional abundance of genes involved in growth-related pathways was lowered within developmental *periods 2* and *3*, indicating an impaired muscle growth performance up to adulthood, mainly compensated by increased body fat accumulation. Indeed, at weaning (28 dpn), offspring of the LP group showed slightly, but significantly, higher fat content and adipocyte size, and still lower muscle fiber numbers. Furthermore, neither body weight at weaning nor body weight at 188 dpn differed significantly among offspring of the LP and AP groups, but visceral and subcutaneous fat content remained higher, while muscle fiber number remained lower in LP than in AP during postnatal life (34, 35). The decreased amount of myofibers formed in response to a maternal LP diet in perinatal piglets (34) may have adverse long-term effects on body composition and metabolic traits (32, 43). Notably, the adjustment of the body composition due to birth weight has been revealed by studies in humans, where the birth weight correlated positively with the lean body mass (11).

Changes in transcript abundance of genes from a number of pathways mirror the compensatory growth in favor of fat accumulation rather than muscle tissue growth. Transcripts of genes assigned to pathways playing a role in general cell growth and proliferation, like RAN signaling, VEGF signaling, and G1/S and G2/S checkpoint regulation, show divergent abundance among dietary groups. In particular, mTOR signaling, an important nutrient-sensing pathway that controls protein synthesis in mammalian cells at the level of translation (16), is decreased within developmental *periods 1* and *2* in LP offspring. This may lead to reduced protein synthesis in LP offspring, though, overall, LP offspring showed equivalent growth rates after weaning. Glucocorticoids act on muscle growth in a catabolic manner, including not only a diminished de novo protein synthesis but also an increased protein degradation (18, 25). In LP sows an increase in plasma cortisol levels was measured in late pregnancy. Furthermore, there were clues for increased concentrations of biologically active cortisol in LP fetuses of 93 dpc, calculated from the free cortisol index (17). This is in line with observed transcriptional alterations of genes associated with the glucocorticoid receptor signaling within developmental *period 1* in LP offspring. The

diet-dependent alteration in glucocorticoid receptor signaling may contribute to hampered skeletal muscle growth.

During postnatal development, pathways related to lipid metabolism were altered. Consistent with these observations, genes associated with lipid metabolism were also altered in rodent LP models at pre- and postnatal stages (2, 7, 12, 20–22). The transcriptional adaptations are in line with the consequences for the offspring's organismal phenotype. Taken together, alterations of genes and pathways, which are associated with lipid metabolism, may be seen as a side-effect in terms of postnatal adaptive responses to the prenatal nutritional environment in LP offspring.

Energy-utilizing and metabolism-regulating pathways displayed expression changes by maternal diet. Various studies demonstrated that maternal gestational LP diets affect both IGF1 and insulin content. Differences between the experimental groups were mainly dependent on the exposure time of the dietary challenge and the ontogenetic stage (3, 4, 6, 14). These diet-dependent modifications may cause impairments in glucose metabolism and growth performance (10, 30, 31). Consistently, our experiment showed alterations of both IGF1 signaling and insulin signaling in skeletal muscle tissue (Fig. 3). The observed transcriptional shift in IGF1 signaling due to maternal LP diet may contribute to IUGR and influence muscle development. Incidentally, LP sows had decreased plasma IGF1 concentration in early and midpregnancy compared with sows fed AP (23). Additionally, genes associated with insulin receptor signaling were biphasically altered in an age-dependent manner in porcine LP offspring. Hence, diet-dependent effects on insulin sensitivity are the most likely result (reviewed in Ref. 8).

However, these transcriptional alterations and their possible phenotypic effects differ from results of other animal experiments using a LP model. Findings in young murine offspring suggest ameliorated insulin sensitivity, including an improved glucose tolerance and reduced insulin concentrations (29, 38). In contrast, our study revealed a lowered transcriptional abundance of genes related to insulin receptor signaling within developmental *period 2* and a trend for increased insulin concentrations at weaning ($P = 0.06$). This suggests that ligand-based signal transduction is less sensitive at weaning in LP offspring. Regarding adult murine LP offspring, the biopositive effects observed in rodents at younger stages were reversed when impaired glucose tolerance and insulin resistance were observed (10, 31). In contrast, our study showed unaltered basal insulin concentrations at 188 dpn and an increased transcriptional abundance of genes related to insulin receptor signaling, indicating an increased signal transduction.

In addition to muscle insulin-dependent metabolism, other pathways related to energy utilization are affected by LP gestation diets. Transcripts of genes associated with oxidative phosphorylation are relatively more prominent in LP offspring at developmental *period 1* as well as at 188 dpn. Regarding previously described health-promoting benefits of an activated mitochondrial system (37), this transcriptional shift could be categorized as a biopositive effect. However, considering that a poor protein supply in utero led to a numerical decrease of myofibers in skeletal muscle in LP (34) the increased transcriptional abundance of genes related to the of OXPHOS may likely reflect compensatory responses. This adaptation suggests that the available mitochondrial capacity of LP offspring warrants the daily high energy demand of skeletal muscle. Therefore, the increased mRNA expression of OXPHOS in LP offspring might not be associated with prevailing biopositive effects.

Conclusion

The longitudinal survey of changes in transcript abundance in skeletal muscle in response to gestational diets with low [LP, 6.5% crude protein (CP)] or adequate protein (AP, 12.1% CP) contents at prenatal (94 dpc), perinatal (1 dpn), juvenile (28 dpn), and adult (188 dpn) developmental stages revealed acute short-term and delayed long-term modulations. In terms of quantity and quality, the transcriptional response to maternal diets was shown to be stage dependent. In our model the gestational LP diet causes only weak acute differences compared with the AP diet. The dietary impact became most obvious as temporal shifts in the transcriptome accumulated in long-term effects that were prominent at the adult stage. Changes in transcript abundance were not persistent in terms of consistent differential expression of genes at all stages. However, genes related to cell cycle were differentially expressed during the developmental phases that were monitored. Differential expression of genes related to growth indicates that the offspring of both groups use different metabolic directions in response to identical nutritional conditions during postnatal life. These alterations might be related to both IUGR and postnatal compensatory effects. The expression profiles indicate adaptive response in terms of compensatory growth, with a slight shift of body composition toward fat accumulation at the cost of muscle growth, which is in line with whole body observations.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.O., E.M., S.P., and K.W. performed experiments; M.O., E.M., S.P., and K.W. analyzed data; M.O., E.M., C.C.M., S.P., and K.W. interpreted results of experiments; M.O. prepared figures; M.O. and K.W. drafted manuscript; M.O., E.M., C.C.M., S.P., and K.W. approved final version of manuscript; E.M., C.C.M., and S.P. edited and revised manuscript; C.C.M. and K.W. conception and design of research.

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3 Discussion

We evaluated hepatic and muscular gene expression profiles of the progeny of sows fed either an isoenergetic maternal low protein (LP), high protein (HP) or adequate protein (AP) diet throughout the whole pregnancy by applying whole-genome microarrays. In order to investigate transcriptional features of developmental nutritional programming we conducted a longitudinal experimental design covering prenatal, perinatal, juvenile and adult ontogenetic stages in a porcine model. The comparisons of the relative mRNA abundances provided an overall view of the developmental plasticity of liver and skeletal muscle tissue. An *in utero* exposure to adverse gestational protein diets revealed diet-, tissue- and stage-dependent gene expression patterns. Because there is no best practice for the analysis of microarray data one section discusses the way it has been done here.

3.1 Advantages and disadvantages of methods used in the microarray workflow

Regarding this project, microarray experiments provided a valuable instrument to gain detailed information about affected gene networks, gene-nutrient interactions and the induction of different phenotypes. Notably, microarrays have a descriptive character and only provide a snap-shot of the transcriptome. Further, the abundance of a measured mRNA is not necessarily accompanied by the relative abundance of the corresponding protein [115, 116].

3.1.1 Quality control, normalisation and filtering of microarray data

It has to be stated, that array selection due to quality control, performed normalisation method and filtering steps might lead to biases in the expression data set with a possible impact on the outcome of statistical parameters like P-value, q-value or Fold change. In order to minimize these biases we applied strict quality criteria and used a strongly recommended normalisation method and filtering steps.
In this study, the quality control was figured out by using 'arrayqualitymetrics', a function implemented in R, to generate various graphics, including boxplots of intensities, NUSE plots (Normalized unscaled standard error), RLE plots (Relative log expression), check for RNA degeneration, percentage of present calls and heatmaps [117]. Within this project, 253 of 264 projected microarrays passed the quality criteria and were used for further calculations (liver tissue: n = 184 of 192, skeletal muscle tissue: n = 69 of 72). The row data were preprocessed using the GC-RMA (GeneChip robust multiarray averaging) algorithm because it is the state-of-the-art normalisation method [118] to estimate gene expression in relation to certain transcript yields in the studied samples (Figure 3.1A). It was proposed to improve the statistical power by reducing the number of statistical tests [119]. Therefore, two filtering steps were performed to skip those probe-sets declared as absent (MAS5 filtering (Microarray suite 5.0)) as well as those probe-sets with a small standard deviation among all arrays included in an analysis (Figure 3.1B). The normalisation and filtering steps chosen in this study led to an independence of standard deviation and a logarithmized expression level (Figure 3.1C).

3.1.2 Statistical parameters and thresholds

In order to elucidate transcriptional alterations in liver and skeletal muscle tissue variance analyses were performed (see section 3.2). As indicated by the distribution of p-values, which reflects the degree of variance between the biological replicates, the maternal diets have an impact on gene expression (Figure 3.1D). Regarding multiple testing problems, corresponding q-values were calculated [120] to estimate the proportion of false positives among all significant hypotheses (Figure 3.1E). Furthermore, a Fold change (FC) was computed to reflect differences in expression levels (Figure 3.1F). The calculated FC in fetuses and offspring were moderate.

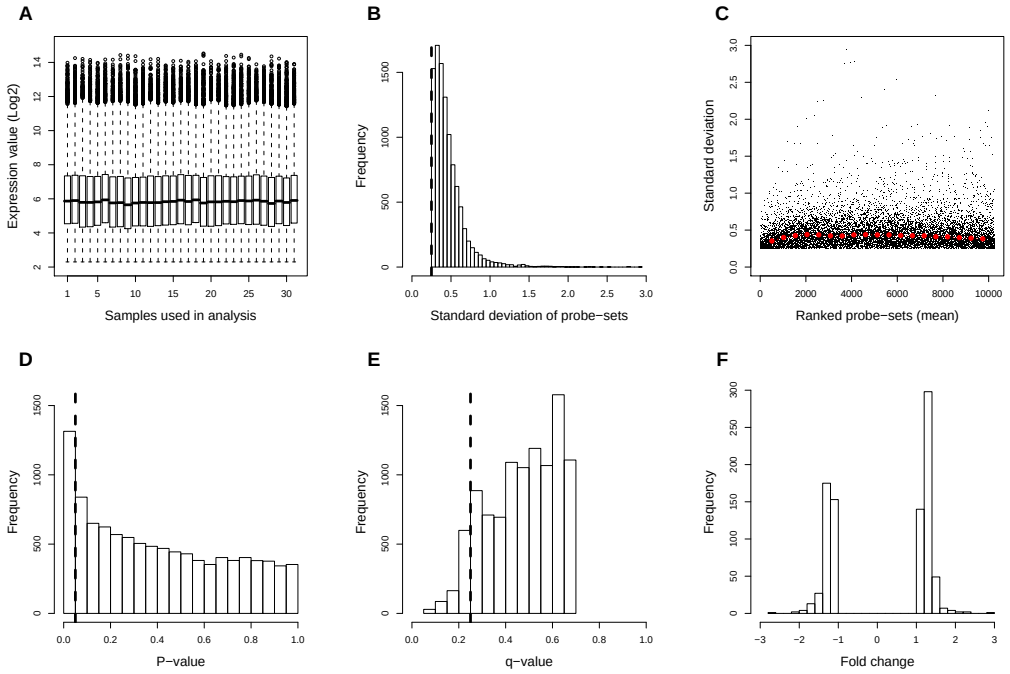

Figure 3.1: Graphics of normalised, transformed (Log2) and filtered data Exemplarily, plots comparing HP vs. AP progeny in liver tissue at stage 1 dpn are shown. A) Transformed intensities (Log2) of arrays used in the analysis. B) Standard deviation of probe-sets among all arrays of the analysis. C) Ranked mean of probe-sets in relation to their standard deviation among all arrays. D) Distribution of p-values. E) Distribution of q-values. F) Distribution of the Fold change (FC) of significantly expressed probe-set ($p \leq 0.05$; $q \leq 0.25$), FC $<$ -1: down-regulated probe-sets, FC $>$ 1: up-regulated probe-sets.

This is in line to microarray experiments investigating dietary interventions [16, 26]. It was suggested that threshold choices may impact the outcome of microarray experiments [121]. Hence within this study, every single statistical calculation considered both the level of significance at $p \leq 0.05$ as well as a moderate threshold to reduce type I errors at $q \leq 0.25$. No cut-off was set for FC.

3.1.3 Annotation and biological interpretation tools

The processing of microarray data requires annotation information which may impact the outcome of a data set. To ensure the advantage of up-to-date information the annotation used in this study was published recently as EnsEMBL Sus scrofa 9, including 20,439 annotated probe-sets [122]. To gain biological information about the interaction of regulated genes due to the dietary treatments, HUGO gene symbols were used to work with data bases like Ingenuity pathway analysis (IPA, Stanford USA) [123] and Database for Annotation, Visualization and Integrated Discovery (DAVID) [124]. It should be noted here that especially IPA focuses rather on human diseases than on pig related developmental processes. Therefore, the interactions presented in the IPA networks are not specific for porcine liver and skeletal muscle tissue. However, IPA is a curated data base which includes recent literature.

3.2 Similarities and differences of the statistical analyses of liver and skeletal muscle tissue

Similarities and differences regarding the computational analyses applied in liver and skeletal muscle tissue occured. The inadequate dietary groups were compared to the AP group in both liver and skeletal muscle tissue. Furthermore, all dietary comparisons were considered as longitudinal studies in both tissues, including prenatal, perinatal, juvenile and adult stages. According to the experimental design, three developmental periods were set and adjacent ontogenetic stages were compared (developmental period I: 94 dpc & 1 dpn; developmental period II: 1 dpn & 28 dpn; developmental period III: 28 dpn & 188 dpn). Hence, the analyses of liver tissue were conducted in three steps, including those samples belonging to a certain developmental period. Due to the reduced sample size, the bioinformatic analyses of skeletal muscle tissue were performed including all ontogenetic stages (Figure 1.1). The performed variance analyses included the factor 'mother' as fixed effect in liver tissue but as random one in skeletal muscle tissue. Moreover, in liver tissue the variable 'weight' was grouped into underweight and overweight progeny at stages 94 dpc and 1 dpn. On the contrary, in skeletal muscle tissue the variable 'weight' was included into the analyses as variation of the mean

(within stage in percent at all stages). Only those factors were maintained within the analyses which showed either a diet-dependent effect itself or within an interaction with another factor. Although only few sex-specific probe-sets exceeded the level of significance within the analyses, the factor 'sex' was kept within the statistical model. However, remarkable effects partially revealed within the interaction 'diet x sex' (Table 3.1). Notably, no batch-effect was detectable within the analyses. Thus, no other factors were included.

3.3 Diet-dependent transcriptional excursions in liver and skeletal muscle tissue along ontogenesis including pre- and postnatal time points

Following *in utero* exposure to adverse gestational protein diets, we found differences in gene expression due to maternal diet, ontogenetic stage and analysed tissue (Figure 3.2).

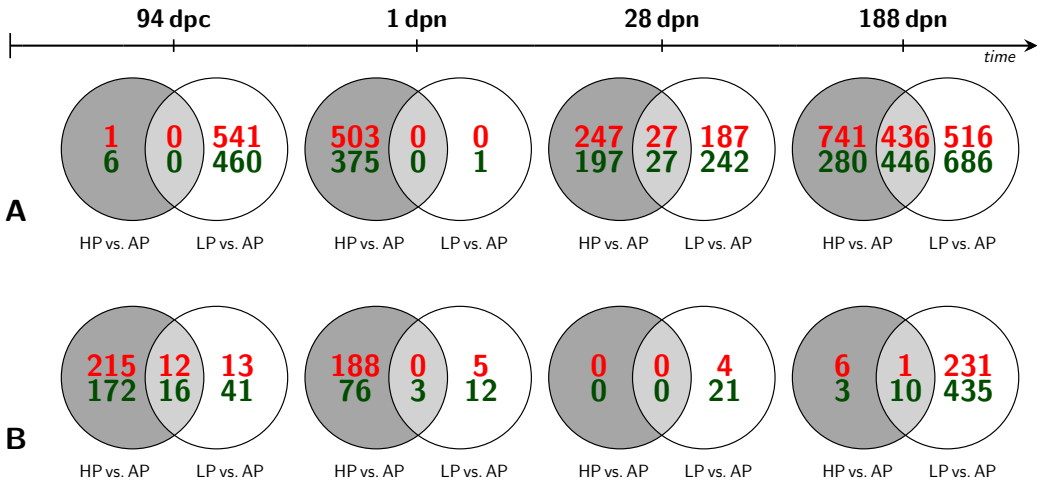

Figure 3.2: Number of diet-dependently regulated probe-sets at prenatal and postnatal ontogenetic stages in liver and skeletal muscle tissue. Significantly regulated probe-sets ($p \leq 0.05$; $q \leq 0.25$) are shown in red (up regulated) and green (down regulated). Gray circles indicate the comparison between offspring of high protein (HP) vs. adequate protein (AP). White circles indicate the comparison between offspring of low protein (LP) vs. adequate protein (AP). (A) Liver tissue, (B) Skeletal muscle tissue.

Our model indicates that gestational LP as well as HP diets affected the expression profiles of the progeny both short-term and long-term. Consequently, the responsiveness and adaptability of the gene expression machinery are affected by acute and chronic environmental stimuli. These diet-specific regulations reflect the high developmental plasticity of mammals, which are able to counterbalance adverse environmental effects like dietary challenges to a large extent. However, one has to consider that the data only provide a snapshot of the transcriptome. Thus, it remains unclear whether transcriptomic differences, observed at pre- and postnatal stages between the experimental groups, were due to chronic long-term effects or due to acute stimulating events in life (e.g. birth or weaning). Thanks to the longitudinal study design, physiological maturation processes were discarded and only those transcriptional regulations, which were private to each dietary group, remained in the analyses. In order to unravel possible diet-specific mechanisms (associated with IUGR, compensatory growth and metabolic disturbances), the comprehensive analyses focused on pathways related to 'Organismal and cellular growth, proliferation and development', 'Cell cycle regulation', 'Energy metabolism', 'Lipid metabolism', and 'Glucocorticoid receptor signaling' in both liver and skeletal muscle tissue (Figure 3.3 and Figure 3.4).

The 'diet-specific expression patterns' and the 'time-delayed transcriptional response to the dietary challenges at early stages' suggest that highly sensitive mechanisms influence the transcriptome profiles in order to establish the most advantageous survival strategies to environmental conditions. According to our analyses, these mechanisms do not primarily impact hierarchically superior genes, encoding transcription factors (TF) and regulatory elements (RE). Therefore, we conclude that the observed transcriptional response was implemented in a gene-specific manner.

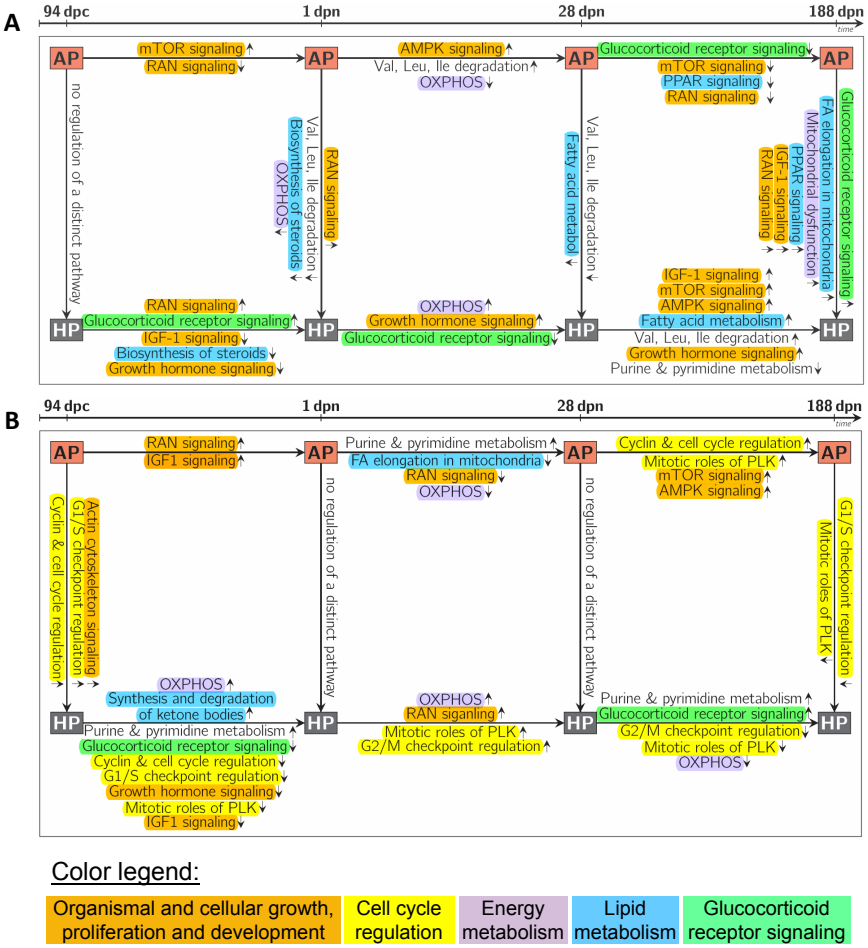

Figure 3.3: Regulated pathways in liver and skeletal muscle tissue due to a maternal high protein diet. Listed pathways between AP stages (red boxes) indicate the appropriate ontogenetic development, which does not take place in the HP offspring (black boxes) at the corresponding developmental period. Pathways between the HP stages indicate processes and metabolic regulations, which occur in the HP offspring but not in the AP offspring in the corresponding developmental period. The differences in gene regulation depending on diet and ontogenetic stage indicate 'Fetal Programming' in terms of developmental and metabolic disorders (arrows between boxes show direction of the comparison; small arrows to top = up-regulated, small arrows to bottom = down-regulated pathways).

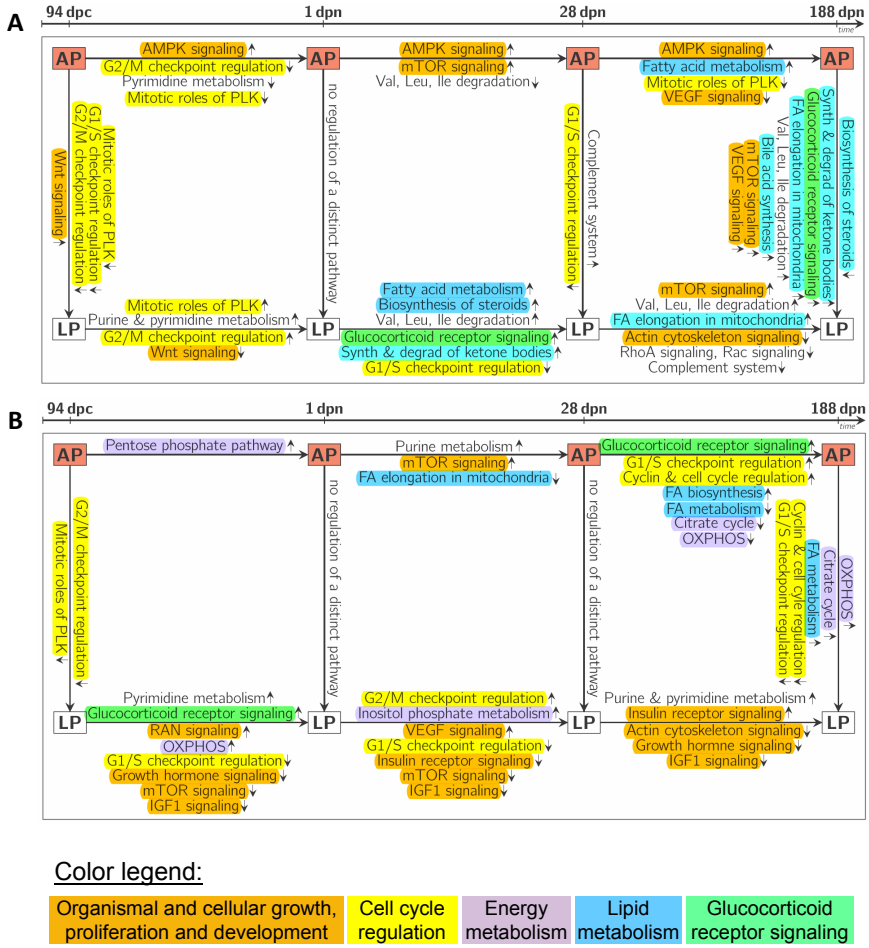

Figure 3.4: Regulated pathways in liver and skeletal muscle tissue due to a maternal low protein diet. Listed pathways between AP stages (red boxes) indicate the appropriate ontogenetic development, which does not take place in the LP offspring (black boxes) at the corresponding developmental period. Pathways between the LP stages indicate processes and metabolic regulations, which occur in the LP offspring but not in the AP offspring in the corresponding developmental period. The differences in gene regulation depending on diet and ontogenetic stage indicate 'Fetal Programming' in terms of developmental and metabolic disorders (arrows between boxes show direction of the comparison; small arrows to top = up-regulated, small arrows to bottom = down-regulated pathways).

3.3.1 No persistent diet-specific regulations were found among ontogenesis

It was proposed that effects mediated by an environmental factor show persistent metabolic modifications until adulthood, including permanent consequences for phenotype [14]. Furthermore, there is evidence that diet-dependent effects on DNA methylation patterns are persistent [96]. This led to the hypothesis that the offspring's transcriptome is persistently regulated by the maternal diet throughout gestation. Due to the longitudinal experimental design of this study, possible persistent patterns in gene regulation were identifiable. However, no single gene was found differentially expressed between the groups at all examined stages. This is consistent with results of dietary interventions published recently, which state that only a few permanent effects on the transcriptome were found [32]. Regarding the analyses, 142 probe-sets were differentially regulated between HP and AP offspring at both stages 1 dpn and 188 dpn in liver tissue. Furthermore, 179 probe-sets between LP and AP offspring differed in their mRNA abundances at both stages 94 dpc and 188 dpn in liver tissue. However, these numbers do not exceed the quantities one would expect to be observed by chance. Notably, on the level of pathways and functional networks mitochondria related genes were found to be diet-dependently regulated between HP and AP offspring at both stages 1 dpn and 188 dpn in liver tissue. Taken together, we observed no persistent transcriptional regulations between stages. This accounts for the huge potential in terms of developmental plasticity of mammals.

3.3.2 Diet-dependent regulations of probe-sets in liver and skeletal muscle tissue

Our longitudinal holistic study design was eligible to trace diet-dependent modulations of the progeny's transcriptome between dietary groups in liver and skeletal muscle tissue along ontogenesis. The liver is the central metabolic organ and its activities are essential for storage, utilization, and partitioning of nutrients. In contrast, skeletal muscle tissue as peripheral tissue acts less metabolic active, due to its structural functions. Therefore, liver tissue of fetuses and offspring might be more prone to effects caused by the maternal dietary intervention than skeletal muscle tissue. This matter is reflected by the number of regulated probe-sets: Liver tissue showed much more regulated probe-sets than skeletal muscle tissue (Figure 3.2). The intensive transcriptional regulations suggest that in pigs liver tissue is the major target of

programmed molecular mechanisms leading to the establishment of a 'metabolic memory'.
At prenatal stage the fetuses were exposed to a serious nutritional challenge that requires short-term adaptive regulations of the transcriptome. Low protein fetuses showed extensively altered mRNA expression profiles in liver tissue, but only relative few regulations in skeletal muscle tissue when compared to AP fetuses. Interestingly, a maternal HP diet did not lead to significant transcriptional changes in porcine fetal liver, but in skeletal muscle tissue altered mRNA expression was observed. Obviously, in porcine fetuses central and peripheral tissues responded in a diet-specific manner. This circumstance might be due to their different responsibilities regarding metabolic activity and structural function.
Because of the adverse intrauterine protein supply, significant transcriptional changes were only found in porcine perinatal HP liver and HP skeletal muscle tissue, but not in LP offspring. However, at the whole-body level both HP and LP progeny was growth restricted [39]. At this time point the piglets were exposed to major nutritional changes: The nutrient supply of the neonatal piglets changed from the parenteral route (via the umbilical cord and some swallowing of amniotic fluid) to enteral nutrition with colostrum (containing compounds new to the individual, e.g. fat, lactose, immunoglobulins) [125]. Obviously, with respect to the amounts of regulated probe-sets, these changes elicited transcriptional adaptations in HP rather than in LP piglets and assigned a programmed respond to new environmental cues. Therefore, it may be easier to deal with an adequate protein supply when exposed to a poorer environment *in utero* than exposed to a richer environment *in utero*.
At weaning, the analyses of liver tissue revealed relatively few amounts of regulated probe-sets in both dietary comparisons (HP vs. AP progeny, LP vs. AP progeny). Additionally, in skeletal muscle tissue hardly any probe-set was found to differ diet-dependently. Thus, in both liver and skeletal muscle tissue HP and LP offspring may have adapted their expression profiles to early postnatal feeding conditions during weaning time. However, the number of differentially expressed probe-sets among the two dietary comparisons in liver tissue increased again at adult stage. Regarding skeletal muscle tissue, transcriptional excursions were mainly observed in LP adult offspring at stage 188 dpn. These transcriptional postnatal regulations were seen as long-term effects. Because the offspring was exposed to normal dietary conditions at juvenile and adult stages those alterations were seen as 'Fetal Programming'.

3.3.3 Diet-dependent regulations of relevant pathways

Limited and excessive maternal protein intakes cumulated in a decreased birth weight of porcine offspring [39], but the analyses of expression profiles revealed a different transcriptional response of both LP and HP progeny. Interestingly, the dietary groups showed a numerically diametric gene expression pattern at early stages (Figure 3.2), which possibly happened due to diet-specific adaptation processes. Also, at juvenile and adult stages, both LP and HP progeny deflected their gene expression in a different manner, because expression patterns only revealed a minor intersection between the dietary groups. Therefore, the analyses of mRNA expression patterns unravelled mainly diet-specific effects along ontogenesis (Figure 3.3 and Figure 3.4). According to the transcriptional analyses, the 'Fetal programming' of the genome warrants adaptation processes regarding compensatory growth, but it probably does that at the expense of a predisposition for metabolic disturbances up to adult stage. Therefore, neither HP nor LP diets are recommendable when fed throughout pregnancy.

3.3.4 Tissue-dependent regulations of relevant pathways

The liver is a central metabolic organ and its activities are essential for storage, utilization, and partitioning of nutrients. Hence, it is probably one of the major targets in terms of 'Fetal Programming'. Additionally, skeletal muscle plays an important role in consuming and storing of energy equivalents and nutrients. It is a peripherial tissue that contributes to the species-typical shape of the body.
The transcriptional response to inadequate maternal diets showed expression patterns depending on the metabolic demand of central and peripheral tissues (Figure 3.3 and Figure 3.4). In liver tissue, the overall synthesis of the expression analysis indicates that the maternal protein supply provokes a 'Programming' of the progeny's genome. The 'Programming' is characterized by the adjustment of energy sensing pathways, energy producing pathways (HP) as well as molecular signaling pathways related to cell cycle regulation, proliferation and maintenance (LP). Furthermore, stage-dependent regulations among the treatment groups reflect compensatory growth that is associated with the observed IUGR in both HP and LP progeny. Moreover, the observed hepatic gene expression patterns point to alterations in the 'Energy metabolism' of

the HP progeny and to a modified 'Lipid metabolism' in both malnourished groups. Taken together, the data support the hypothesis of 'diet-induced IUGR and compensatory growth followed by a predisposition for metabolic disorders in adulthood' in both HP and LP progeny. Due to maternal protein diets both HP and LP progeny was growth retarded at birth but archieved their organismal weight at juvenile and adult stages [20, 126]. Particularly in skeletal muscle tissue the growth performance is very important, because it accounts for a high percentage of body mass. Thus, it has to be expected that growth associated pathways play an important role in skeletal muscle tissue. Consistently, pathways summarised as 'Organismal and cellular growth, proliferation and development' and 'Cell cycle regulation' were diet-dependently regulated at various time points in skeletal muscle tissue in both treated groups. These findings were in line with with recent programming studies which suggest that the regulation of the cell cycle, as well as mechanisms cumulating in tissue remodelling, may be part of the primary pathological changes occurring due to inadequate maternal protein diets [16, 127]. Therefore, it is necessary to focus on epigenetic processes which investigate key components of the cell cycle regulation like cyclins and cyclin-dependent kinases.

3.3.5 Stage-dependent regulations of relevant pathways

The performed microarray experiment revealed that gestational diets affect the expression patterns in a short-term and in a long-term manner in both HP and LP offspring. The diet-dependent effects suggest chronic and acute environmental stimuli, leading to stage-specific responsiveness and adaptability of the gene expression machinery. On the one hand the investigated fetuses and offspring had to deal with the intrauterine exposure to the adverse gestational protein diets and the nutritional challenge regarding the unexpected adequate protein supply postnatally. On the other hand the animals were exposed to different nutrient supplies, including parenteral nutrition via the umbilical cord, enteral nutrition with colostrum, and afterwards, barley and soybean meal-based diets.
Due to gestational protein diets the hepatic transcriptome of HP and LP offspring was affected oppositionally at fetal and perinatal stages (Figure 3.2). At stages 28 dpn and 188 dpn the dietary comparisons between either HP vs. AP and LP vs. AP showed an almost similar amount of regulated probe-sets in liver tissue, which were partly concordant regarding their direction of regulation. The programmed response for new environmental cues was stage-

independent with regard to pathways associated to compensatory growth ('Organismal and cellular growth, proliferation and development' and 'Cell cycle regulation') and 'Glucocorticoid receptor signaling' in both investigated tissues in HP and LP progeny (Figure 3.3 and Figure 3.4). In contrast, metabolic pathways represented in 'Lipid metabolism' and 'Energy metabolism' were diet-dependently regulated rather at later stages. Taken together, the diet-dependent expression patterns suggest that both HP and LP progeny counterbalanced the IUGR at the expense of their metabolic health.
In skeletal muscle tissue, the observed transcriptional regulations were transient and differed between fetuses and offspring. Furthermore, no genes were assigned to distinct regulatory pathways in perinatal and weaning piglets in the treated groups. This is consistent with recent findings when dietary modifications during pregnancy have been investigated in skeletal muscle tissue of young adult rats [26]. However, in skeletal muscle tissue the mRNA abundances of both HP and LP fetuses and offspring revealed associations with the observed IUGR along ontogenetic development.

3.3.6 Impact of other factors

Some experiments in rodents showed that sex has an impact on diet-induced IUGR [82], postnatal growth performance [98], transcriptional responses [27, 88] and hormone levels [128]. Thus, an animal selection, which was balanced for sex at all stages, was investigated in our study. However, with respect to the performed variance analyses the factor 'sex' only provoked a transcriptional response of less than 50 probe-sets per analysed stage (Table 3.1). Thus, the different duration of the gestation (i.e the dietary intervention) as well as the disparate degree of maturation of rodents and pigs at birth might contribute to the different findings. Notably, the interaction between 'diet $\times$ sex' revealed a stage-specific hepatic mRNA expression in our study. Out of 48 possible entries only 5 entries showed a substantial amount of regulated probe-sets, due to dietary comparison, sex-specific dietary regulation and ontogenetic stage.

Table 3.1: Regulated probe-sets due to the factors 'sex' and 'diet × sex' in liver tissue at different ontogenetic stages. The impact of factor 'sex' is marginal at all analysed ontogenetic stages. The interaction between 'diet × sex' revealed a stage-specific mRNA expression, that is independent of distinct comparisons.

Item	Ontogenetic stage			
	94 dpc	**1 dpn**	**28 dpn**	**188 dpn**
HP vs. AP				
'sex'	12	10	35	10
'diet × sex'				
AP male vs. AP female	13	9	4	15
AP male vs. HP male	0	0	0	12
AP male vs. HP female	12	15	2,220	248
AP female vs. HP male	15	11	13	12
AP female vs. HP female	2	401	4	44
HP male vs. HP female	1	9	8	0
LP vs. AP				
'sex'	17	11	13	10
'diet × sex'				
AP male vs. AP female	0	13	12	12
AP male vs. LP male	2	0	1	16
AP male vs. LP female	32	9	10	42
AP female vs. LP male	16	12	1,674	47
AP female vs. LP female	2	1	1	858
LP male vs. LP female	14	10	11	15

According to the performed variance analyses the effect 'mother' explained a substantial amount of the variances in both liver and skeletal muscle tissue. Furthermore, the dominant impact of the genetical background was apparent in hierarchical cluster analyses. Thus, studies investigating dietary treatments should contain samples originating from many different litters.

3.4 Implications and Outlook

We could show that maternal protein diets fed throughout pregnancy impact the gene expression of the fetuses and offspring up to adult stage. With regard to the stage-specific transcriptional response and the lack of persistent diet-dependent expression patterns it has to be stated that the proximate causes of the observed transcriptional changes remain to be determined. Therefore, the results of the transcriptome profiles have to be related to complementary data from collaborators involved in the project FEPROeXPRESS. The synthesis of the available experimental data, including DNA methylation pattern, transcriptome, proteome and metabolome profiles as well as data investigating the hypothalamic-pituitary-adrenal axis, will contribute to describe a sophisticated picture of the phenomenon 'Fetal Programming' in a porcine model.

Our analyses indicate that the transcriptional response due to prenatal nutritional challenges does not primarily refer to the level of hierarchically superior genes, encoding transcription factors of regulatory networks. Along with the finding that dietary interventions revealed gene-specific regulations, further research should focus on epigenetic gene-specific modifications to be able to explain the regulations of signaling pathways and metabolic routes.

Because effects of intrauterine malnutrition are studied to unravel the origin of adult diseases it is important to focus on mitochondrial metabolism and mitochondrial DNA (mtDNA), because mitochondria are known to have a vitally importance in cell metabolism and health. Furthermore, mtDNA is exclusively bequeathed by the mother. Taken together, diet-dependent epigenetic processes of genes encoded by mtDNA, might play a crucial role in terms of compensatory growth and metabolic balance.

4 Summary

Adverse environmental conditions during fetal development provoke an intrauterine adaptive response termed 'Fetal Programming', characterized by long-term consequences for growth, function and structure of various tissues of the progeny. Inadequate maternal protein supply during gestation represents an environmental factor, that is known to affect physiological signaling pathways and impacts the phenotype of the progeny in animal models and in humans. Hypothesising that the progeny's transcriptome is persistently altered by maternal diets, we used a porcine model to monitor the longitudinal expression changes in liver and skeletal muscle tissue to identify pathways relevant to the fetal initiation of postnatal growth and development. Because pigs share many similarities with humans regarding genome, anatomy, physiology, and metabolism they provide a good animal model. Throughout the whole pregnancy nulliparous German Landrace sows were fed one of three isoenergetic diets differing in their protein:carbohydrate ratio, resulting in a low protein diet (LP: 6.5 % crude protein, protein:carbohydrate ratio 1:10), a high protein diet (HP: 30 % crude protein, protein:carbohydrate ratio 1:1.3) and an adequate protein diet (AP: 12.1 % crude protein, protein:carbohydrate ratio 1:5). The progeny of all dietary groups was nursed by foster sows that received a standard diet. Postweaning, the progeny was fed standard diets *ad libitum*. The progeny's liver (n = 192) and skeletal muscle tissue (*M. longissimus dorsi*; n = 72) were collected at 94 days post conception (dpc) and 1, 28, and 188 days post natum (dpn) for expression profiling using genome-wide GeneChip® Porcine Genome Arrays. The resulting dietary comparisons (HP vs. AP and LP vs. AP, respectively) were processed separately in both analysed tissues, investigating prenatal, perinatal, juvenile, and adult ontogenetic stages. The analyses included comparisons between dietary groups within ontogenetic stages as well as comparisons between ontogenetic stages within dietary groups to separate diet-specific transcriptional changes and physiological maturation processes. Microarray data were GC-RMA (GeneChip robust multiarray averaging) normalized and filtered by both MAS5 (Microarray suite 5.0; present call $\geq$ 50 % per dietary

group, stage and tissue) and standard deviation (SD $\geq$ 0.25). Transcripts were considered as significant at p $\leq$ 0.05 (q $\leq$ 0.25). Transcriptional responses were revealed using a variance analysis, including effects of 'diet', 'mother', 'sex', 'stage', and 'weight'. The biological analysis was based on EnsEMBL Sus scrofa 9 and Ingenuity Pathway Analysis. An *in utero* exposure to adverse gestational protein diets revealed a 'Programming' of the offspring's genome in a diet-, tissue- and stage-dependent manner. Depending on the maternal dietary exposure during fetal time, the progeny showed a differential expression of genes related to LIPID METABOLISM (e.g. fatty acid metabolism, fatty acid biosynthesis, biosynthesis of steroids, synthesis and degradation of ketone bodies, fatty acid elongation in mitochondria, bile acid synthesis, PPAR signaling), CELL CYCLE REGULATION (e.g. mitotic roles of PLK, G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, cyclin and cell cycle regulation), ENERGY METABOLISM (e.g. oxidative phosphorylation, mitochondrial dysfunction, citrate cycle, pentose phosphate pathway, inositol phosphate metabolism), ORGANISMAL AND CELLULAR GROWTH (e.g. Ran signaling, growth hormone signaling, mTOR signaling, IGF1 signaling, insulin receptor signaling, actin cytoskeleton signaling, VEGF signaling, AMPK signaling, Wnt signaling), and GLUCOCORTICOID RECEPTOR SIGNALING. A noticeable amount of altered transcript abundances was due to the variance component 'mother', while the factor 'sex' was less important. No single gene was found differentially expressed between the dietary groups along all examined stages. The overall synthesis of the expression analysis reflects compensatory growth in both HP and LP progeny that is associated with the observed intrauterine growth retardation in the treated groups. Thus, the transcriptional responses in both HP and LP progeny were interpreted as the molecular equivalent to developmental plasticity which accounts for adaptation and maintenance of the organismal phenotype and affects signaling pathways related to energy utilisation. Obviously, the 'Fetal Programming' of the genome warrants adaptation processes regarding to compensatory growth, probably at the expense of a predisposition for metabolic disturbances up to adult stage. Hence, neither HP nor LP diets are advisable when fed throughout pregnancy. The analysis indicates that the transcriptional response due to prenatal nutritional challenges does not primarily refer to the level of hierarchically superior genes, encoding transcription factors of regulatory networks. Therefore, further research should focus on epigenetic gene-specific modifications to find a way to explain the primary mechanisms of 'Fetal Programming'.

Ungünstige Umwelteinflüsse während der fetalen Entwicklung bewirken eine intrauterine adaptive Antwort mit langfristigen Konsequenzen auf Wachstum, Funktion und Struktur verschiedener Gewebe der Nachkommen. Diese Zusammenhänge werden als 'Fetale Programmierung' bezeichnet. So stellt eine unangepasste maternale Proteinversorgung während der Gestation einen Umweltfaktor dar, der sowohl physiologische Signalwege beeinflusst als auch phänotypische Anpassungen bei Nachkommen von Mensch und Tier hervorruft. In der vorliegenden Arbeit wurde die Hypothese einer persistenten Transkriptomregulation der Nachkommen in Abhängigkeit von der mütterlichen Diät in einem porcinen Model bearbeitet. Ziel war es, molekulare Signalwege und Mechanismen mit Relevanz zur fetalen Initiierung von postnatalem Wachstum und Entwicklung zu identifizieren. Da Menschen und Schweine viele Gemeinsamkeiten hinsichtlich Genom, Anatomie, Physiologie und Metabolismus aufweisen, stellen Schweine für humane Fragestellungen ein relevantes Tiermodel dar. Jungsauen der Deutschen Landrasse wurden während der gesamten Trächtigkeit mit einer von drei isoenergetischen Diäten ernährt, die sich in ihrem Protein:Kohlenhydrat-Verhältnis unterschieden. Die experimentellen Gruppen wurden als Niedrigproteindiät (LP, 6% Rohprotein, Protein:Kohlenhydrat 1:10), Hochproteindiät (HP, 30% Rohprotein, Protein:Kohlenhydrat 1:1,3) und adäquate Proteindiät (AP, 12% Rohprotein, Protein:Kohlenhydrat 1:5) bezeichnet. Die Nachkommen wurden von Ammensauen gesäugt, welche mit Standardfutter ernährt wurden. Nach dem Absetzen erhielten die Nachkommen Standardfutter *ad libitum*. Der Nachkommenschaft wurde Leber ($n = 192$) und Muskelgewebe (*M. longissimus dorsi*; $n = 72$) am 94. Tag der Trächtigkeit (dpc) sowie am 1., 28., und 188. Lebenstag (dpn) entnommen. Die hepatischen und muskulären Genexpressionsprofile der Nachkommen wurden mit Hilfe genomweiter Mikroarrays (*GeneChip® Porcine Genome Arrays*) abgebildet. Die sich ergebenden Diätvergleiche (HP vs. AP und LP vs. AP) wurden in beiden untersuchten Geweben über alle ontogenetischen Zeitpunkte separat ausgewertet. Durch das longitudinale Studiendesign konnten sowohl Vergleiche zwischen Diätgruppen innerhalb der ontogenetischen Stadien als auch Vergleiche zwischen ontogenetischen Stadien innerhalb der einzelnen Diätgruppen vorgenommen werden. Auf diese Weise war es möglich, Diät-spezifische transkriptionelle Änderungen von physiologischen Reifungsprozessen zu unterscheiden. Die Mikroarraydaten wurden mit dem GC-RMA Algorithmus (engl. *GeneChip robust multiarray averaging*) normalisiert und anhand von MAS5 (engl. *Microarray suite 5.0; present call* $\geq 50\%$ per Dietgruppe, Stadium und Gewebe) und der Standardabweichung (SD ≥ 0.25) gefiltert. Das Signifikanzniveau wurde auf $p \leq 0.05$ ($q \leq 0.25$) festgelegt. Die transkriptionellen Ausprägungen wurden durch eine Varianzanalyse mit den Komponenten 'Diät', 'Mutter', 'Geschlecht', 'Stadium' und 'Gewicht' bestimmt. Die Interpretation der biologischen

Prozesse basierte auf EnsEMBL Sus scrofa 9 and *Ingenuity Pathway Analysis*. Die *in utero* Exposition zu nicht bedarfsgerechten maternalen Proteindiäten zeigte eine 'Programmierung' des Genoms der Nachkommenschaft in Abhängigkeit von Diät, Gewebe und ontogenetischem Stadium. Die Diät-abhängigen transkriptionellen Regulationen in den Nachkommen umfassten Änderungen des LIPID METABOLISMUS (z.B. Metabolismus der Fettsäuren, Biosynthese der Fettsäuren, Biosynthese der Steroide, Synthese und Abbau der Ketonkörper, Fettsäurenverlängerung in Mitochondrien, Synthese der Gallensäuren, PPAR Signalweg), der ZELLZYKLUS REGULATION (z.B. Mitotische Rolle der PLK, Regulation des G1/S Kontrollpunktes, Regulation des G2/M DNA Schäden Kontrollpunktes, Zykline und Zellzyklusregulation), des ENERGY METABOLISMUS (z.B. Oxidative Phosphorylierung, Mitochondriale Dysfunktion, Zitratzyklus, Pentosephosphat Signalweg, Metabolismus der Inositolphosphate), des ORGANISMISCHEN UND ZELLULÄREN WACHSTUMS (z.B. Ran Signalweg, Somatotropin Signalweg, mTOR Signalweg, IGF1 Signalweg, Insulinrezeptor Signalweg, Aktin-Zytoskelett Signalweg, VEGF Signalweg, AMPK Signalweg, Wnt Signalweg), und des GLUKOKORTIKOIDREZEPTOR SIGNALWEGES. Dabei erwies sich der Muttereffekt als wichtige Varianzkomponente, wohingegen das Geschlecht nur marginale Einflüsse zeigte. Es wurden keine Gene gefunden, die in allen untersuchten ontogenetischen Stadien eine differentielle Auslenkung zwischen den Diätgruppen aufwiesen. Die Gesamtdarstellung der Expressionsanalyse zeigte in den Nachkommen der HP und LP ernährten Sauen Hinweise auf kompensatorische Wachstumsprozesse, die mit der beobachteten intrauterinen Wachstumsretardierung der Diätgruppen assoziiert sind. Daher wurde die transkriptionelle Auslenkung der HP und LP Nachkommen als molekulares Äquivalent einer Entwicklungsplastizität interpretiert, welche für die Adaptation und den Erhalt des rassetypischen Phänotyps sorgt und daneben Signalwege der Energieausnutzung beeinflusst. Offensichtlich garantiert die 'Fetale Programmierung' des Genoms ein rassespezifisches Wachstum, welches mit einer Prädisposition für metabolische Störungen bis zum Erwachsenenalter einhergeht. Daher ist weder eine Fütterung von HP noch von LP Diäten während der Trächtigkeit empfehlenswert. Weiterhin zeigt die Analyse, dass die transkriptionelle Auslenkung aufgrund pränataler nutritiver Herausforderungen nicht primär von hierarchisch übergeordneten Genen gesteuert wird, wie etwa von Transkriptionsfaktoren regulativer Netzwerke. Um die primären Mechanismen der 'Fetalen Programmierung' zu erklären, sollten weitere Forschungsaktivitäten auf epigenetische, Gen-spezifische Modifikationen fokussieren.

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A Enclosure 1

A.1 List of abbreviations

ACC	Acetyl coenzyme A carboxylase
AgRP	Agouti-related protein
AGT	Angiotensinogen
Akt	Protein kinase B
AMP	5' Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
AP	Adequate protein
AT1R	Angiotensin 1 receptor
AT2R	Angiotensin 2 receptor
ATP6	ATP synthase F0 subunit 6
Bax	B-cell lymphoma 2-associated X protein
CAT	Catalase
Cav-1	Caveolin-1
CCND1	Cyclin D1
CDKN1a	Cyclin-dependent kinase inhibitor 1A
cDNA	complementary DNA
cRNA	complementary RNA
DAVID	Database for Annotation, Visualization and Integrated Discovery
CFD	Adipsin
c-Fos	FBJ murine osteosarcoma viral oncogene homolog
ChREBP	Carbohydrate responsive element-binding protein
COX1	Cyclooxygenase-1
CPT1	Carnitine palmitoyltransferase 1
CuZnSOD	Superoxide dismutase [Cu-Zn]
DAT	Dopamine active transporter
DM	Dry mass
DNA	Deoxyribonucleic acid

DNMT1	DNA methyltransferase 1
DNMT3a	DNA methyltransferase 3a
DOHaD	Developmental Origin of Health and Disease
dpc	lat. *dies post conceptionem*
dpn	lat. *dies post natum*
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acid
FAS	Fatty acid synthase
FC	Fold Change
GAL	Galanin
GAL2R	Galanin 2 receptor
GCK	Glucokinase
GC-RMA	GeneChip robust multiarray averaging
GH	Growth hormone
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT3	Glucose transporter 3
GLUT4	Glucose transporter 4
GPDH	Glycerol 3-phosphate dehydrogenase
GPx1	Glutathione peroxidase 1
GR	Glucocorticoid receptor
GS	Glucogen synthase
HGF	Hepatic growth factor
HP	High protein
HSD11B1	11β-hydroxysteroid dehydrogenase 1
HSD11B2	11β-hydroxysteroid dehydrogenase 2
HUGO	Human genome organisation
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGF-2R	Insulin-like growth factor-2 receptor
IGFBPs	Insulin-like growth factor binding proteins
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis

IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
IU	International units
IUGR	Intrauterine growth retardation
IVGTT	Intravenous glucose tolerance test
JAK	Janus kinase
LDL	Low density lipoprotein
LP	Low protein
LPK	Liver pyruvate kinase
LRH	Liver receptor homolog-1
MALDI-TOF-MS	Matrix assisted laser desorption ionization - Time of flight - Mass spectrometry
MAS5	Microarray suite 5.0
MCAD	Medium chain acyl-CoA dehydrogenase
MDH	Malate dehydrogenase
ME	Metabolizable energy
MnSOD	Superoxide dismutase 2, mitochondrial
MOR	μ-opioid receptor
mRNA	messanger RNA
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
Na/K-ATPase	Sodium/potassium adenosine triphosphatase
ND4L	Nicotinamide adenine dinucleotide-ubiquinone oxidoreductase chain 4L
NEFA	Not esterified fatty acids
NFkB	Nuclear factor κ-light-chain-enhancer of activated B cells
NPY	Neuropeptide Y
NRF1	Nuclear respiratory factor 1
NUSE	Normalized unscaled standard error
PLIER	Probe logarithmic intensity error estimation
OGTT	Oral glucose tolerance test
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction

PENK	Preproenkephalin
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PKCζ	Protein kinase Cζ
PPARα	Peroxisome proliferator-activated receptor α
PPARβ	Peroxisome proliferator-activated receptor β
PPARδ	Peroxisome proliferator-activated receptor δ
PPARγ	Peroxisome proliferator-activated receptor γ
PRKAA2	5'-AMP-activated protein kinase catalytic subunit α-2
qRT-PCR	quantitative Real-Time PCR
Rac	Ras-related C3 botulinum toxin substrate
Ran	Ras-related nuclear protein
RE	Regulative Element
REN	Rennin
RETN	Resistin
RLE	Relative log expression
RMA	Robust multiarray averaging
RNA	Ribonuclein acid
ROS	Reactive oxygen species
S6K	S6 kinase
Sirt1	Sirtuin 1
SOCS-3	Suppressor of cytokine signaling 3
SOD1	Superoxide dismutase [Cu-Zn]
SOD2	Superoxide dismutase 2, mitochondrial
SPARC	Secreted protein acidic and rich in cysteine
SREBP-1c	Sterol regulatory element-binding protein-1c
STAT	Signal transducer and activator of transcription
TAG	Triacylgylycerides
TF	Transcription factor
Tfam	Transcription factor A, mitochondrial
TFBS	Transcription factor binding site
TNFα	Tumor necrosis factor α
TR-β1	Thyroid receptor 1β

UCP-2	Uncoupling protein-2
UCP-3	Uncoupling protein-3
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor
vsn	Variance stabilisation and normalisation
VTA	Ventral tegmental area

A.2 Composition of diets

Table A.1: Main ingredients and nutrient composition of the experimental diets[1]. Taken from Sarr et al., 2010 [129].

Item	**Maternal diet**		
	HP	**AP**	**LP**
Main ingredients, g/kg DM			
Corn (Maize)	10.59	270.00	107.66
Barley	208.50	415.00	140.00
Soybean meal	590.00	75.00	-
Soybean hulls	72.00	135.00	200.00
Sugar beet pellets, dried	53.00	55.00	62.00
Corn starch	4.50	-	427.18
Linseed, broken	16.50	4.50	7.50
Beet succrose	10.00	0.50	7.50
Soybean oil	9.00	20.00	22.00
Mineral and vitamin mix [2,3]	25.00	25.00	25.00
L-Tryptophan	-	-	0.29
DL-Methionine	0.49	-	0.06
L-Isoleucine	-	-	0.06
L-Leucine	0.42	-	0.39
L-Phenylalanine	-	-	0.31
Chemical composition, g/kg DM			
Crude protein	300	127	65
Ether extract	37	37	29
Crude fiber	103	74	89
Nutritional value			
ME, MJ/kg DM	13.90	13.30	13.50

[1] Diets with high (HP), adequate (AP) or low (LP) protein content were formulated in the Leibniz Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany.
[2] The following amounts of vitamins per kg diet were supplied: 12,500 UI of vitamin A, 1,250 UI of vitamin D_3, 37.5 mg of vitamin E, 1.875 mg of vitamin K_3, 2.5 mg of vitamin B_1, 6.25 mg of vitamin B_2, 3.75 mg of vitamin B_6, 25 μg of vitamin B_{12}, 31.25 mg of niacin, 75 μg of biotin, 0.312 mg of folic acid, 15.625 mg of pantothen and 125 mg of choline.
[3] The following amounts of minerals per kg diet were provided: 5869 mg of calcium, 1625 mg of phosphorus, 1250 mg of sodium, 250 mg of magnesium, 125 mg of iron, 125 mg of copper, 178.75 mg of zinc, 75 mg of manganese, 0.5 mg of cobalt, 0.75 mg of iodine and 0.25 mg of selenium.

Table A.2: Composition of standard diets for post-weaning, growing and finishing periods[1]. Taken from Sarr et al., 2010 [129].

Item	**Growth stage**				
	29 to 32 dpn	**33 to 48 dpn**	**49 to 76 dpn**	**77 to 105 dpn**	**106 to 188 dpn**
Chemical composition, g/kg DM					
Crude protein	190	180	178	175	167
Ether extract	75	66	45	36	23
Crude fiber	30	40	40	40	45
Total ash	nd	60	60	65	50
Lysine	15.0	13.5	12.1	11.5	9.5
Energy, MJ ME/kg	15.0	14.0	13.8	13.6	13.0
Vitamins					
Vitamin A, UI/kg	15,000	15,000	15,000	15,000	10,000
Vitamin D_3, UI/kg	2,000	2,000	2,000	1,500	1,000
Vitamin E, mg/kg	100	100	100	80	60
Minerals, % DM					
Ca	0.70	0.75	0.75	0.75	0.65
P	0.60	0.58	0.58	0.55	0.50
Na	0.25	0.24	0.24	0.20	0.20
Cu, mg/kg	150	150	150	165	150

[1] The following main ingredients were used according to the growth stages: barley, corn (maize), wheat flour, soybean (meal, roasted, or concentrate), milk powder, molasses, feed sugar, whey powder, oils

originated from plants or fish, oat flakes, potato proteins, calcium carbonate, monocalcium phosphate, sodium chloride, pentahydrate, DL-methionine, L-lysinemonohydro chloride, L-threonine and DL-citric acid, and copper sulfate. dpn = days post natum, nd = not determined.

B Enclosure 2

B.1 Acknowledgement

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B.2 Declaration

Hiermit versichere ich, die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben.

Rostock, 5. April 2012

Michael Oster

B.3 List of publications, talks, and posters

[1] M. Oster, E. Murani, C. Metges, S. Ponsuksili, and K. Wimmers, Transcriptional response of skeletal muscle to a low protein gestation diet in porcine offspring accumulates in growth- and cell cycle-regulating pathways, Physiol Genomics **44(16)**, 811–8 (2012).

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[3] M. Oster, E. Murani, C. Metges, S. Ponsuksili, and K. Wimmers, A low protein diet during pregnancy provokes a lasting shift of hepatic expression of genes related to cell cycle throughout ontogenesis in a porcine model, BMC Genomics **13(1)**, 93 (2012).

[4] M. Oster, E. Murani, C. Metges, S. Ponsuksili, and K. Wimmers, A high protein diet during pregnancy affects hepatic gene expression of energy sensing pathways along ontogenesis in a porcine model, PlosOne **6(7)**, e21691 (2011).

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[6] M. Oster, E. Murani, C. Metges, S. Ponsuksili, and K. Wimmers, A high or low protein diet during pregnancy affects hepatic gene expression along ontogenesis in a porcine model, in *International OSKAR KELLNER SYMPOSIUM 2011 - Metabolic Flexibility in Animal and Human Nutrition, Warnemuende, 9 - 11 September*, 2011.

[7] M. Oster and K. Wimmers, FEPROeXPRESS: Modulation der hepatischen Genexpression bei Nachkommen durch die maternale Proteinaufnahme waehrend der Traechtigkeit, in *FUGATO-Statusseminar, Kassel, 09 - 10 February*, 2011.

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[10] M. Oster and K. Wimmers, Einfluesse maternaler Proteinaufnahmen waehrend der Traechtigkeit auf die Genexpression in Lebergewebe beim neugeborenen Ferkel, in *FUGATO-Statusseminar 2009, Kassel, 14 - 15 October*, 2009.

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[12] C. Metges, M. Peters, H. Sauerwein, A. Ooster, S. Ponsuksili, S. Altmann, M. Oster, C. Rehfeldt, and K. Wimmers, Fetal Programming - Muetterliche Ernaehrung und Merkmalsauspraegung der Nachkommen beim Schwein, GENOMXPRESS **9 (1)**, 11–3 (2009).

[13] M. Oster and K. Wimmers, Einfluesse des maternalen Nahrungsproteingehaltes auf die Genexpression beim Schwein, in *Day of Doctoral students, FBN Dummerstorf, 12 November*, 2008.