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Microarray gene expression profiles of fasting induced changes in liver and adipose tissues of pigs expressing the melanocortin-4 receptor D298N variant

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Lkhagvadorj S, Qu L, Cai W, Couture OP, Barb CR, Hausman GJ, Nettleton D, Anderson LL, Dekkers JC, Tuggle CK. Microarray gene expression profiles of fasting induced changes in liver and adipose tissues of pigs expressing the melanocortin-4 receptor D298N variant. *Physiol Genomics* 38: 98–111, 2009. First published April 14, 2009; doi:10.1152/physiolgenomics.90372.2008.—Transcriptional profiling coupled with blood metabolite analyses were used to identify porcine genes and pathways that respond to a fasting treatment or to a D298N missense mutation in the melanocortin-4 receptor (MC4R) gene. Gilts (12 homozygous for D298 and 12 homozygous for N298) were either fed ad libitum or fasted for 3 days. Fasting decreased body weight, backfat, and serum urea concentration and increased serum nonesterified fatty acid. In response to fasting, 7,029 genes in fat and 1,831 genes in liver were differentially expressed (DE). MC4R genotype did not significantly affect gene expression, body weight, backfat depth, or any measured serum metabolite concentration. Pathway analyses of fasting-induced DE genes indicated that lipid and steroid synthesis was downregulated in both liver and fat. Fasting increased expression of genes involved in glucose sparing pathways, such as oxidation of amino acids and fatty acids in liver, and in extracellular matrix pathways, such as cell adhesion and adherens junction in fat. Additionally, we identified DE transcription factors (TF) that regulate many DE genes. This confirms the involvement of TF, such as PPARG, SREBF1, and CEBPA, which are known to regulate the fasting response, and implicates additional TF, such as ESR1. Interestingly, ESR1 controls several fasting induced genes in fat that are involved in cell matrix morphogenesis. Our findings indicate a transcriptional response to fasting in two key metabolic tissues of pigs, which was corroborated by changes in blood metabolites, and the involvement of novel putative transcriptional regulators in the immediate adaptive response to fasting.

transcription; ESR1; MC4R; feed deprivation

ENERGY HOMEOSTASIS IS AN ESSENTIAL physiological component of all living organisms and when disrupted can lead to complicated conditions such as diabetes, obesity, and anorexia. The pig is a major source of human food worldwide and is also well suited as a model for investigations into human homeostatic mechanisms (54, 63). Thus, understanding the mechanisms that regulate food intake can contribute knowledge relevant to human health and improve agricultural economy, as feed is the largest variable cost in pork production, ranging from 57 to 68% of the total variable cost (29, 44).

In humans and rodents, the melanocortin system plays a pivotal role in food intake regulation. In particular, the melanocortin-4 receptor (MC4R), a G protein-coupled seven-transmembrane receptor, integrates and relays key signals controlling food intake (2, 78). In pigs, central administration of the MC4R agonist NDP-MSH suppresses feed intake (4), and a missense variant N298 of the MC4R gene was associated with increased feed intake, growth, and backfat (41). Studies on the effects of N298 on MC4R function indicate that the D298 variant may be required for normal receptor signaling (42). Interestingly, HEK-293 cells transfected with the D298 variant stimulated cAMP production in response to NDP-αMSH, but no stimulation was observed for the N298 variant in this model system (42). These reports indicate that the role of MC4R in food intake control extends to the porcine species.

Recent advances have been made in swine transcriptomics (23, 74), where transcriptional profiling was successfully employed to probe several porcine tissues such as adipose (31), muscle (50), lung (82), and neural (59) tissues. Studies with rodent models have reported transcriptional responses to fasting in heart (70), hypothalamus (48), muscle (73), liver, and adipose (7, 49, 55) tissues. Several groups have investigated the roles of specific metabolic genes during fasting (5, 28, 67), but global transcriptional profiling of the fasting response has not been reported in the porcine species. Fasting studies in rodent species have also not included global analysis of key regulatory transcription factors (TF) that may be responsible for the differentially expressed (DE) genes. Several TF and nuclear receptors are known to mediate the fasting response (13, 18, 46), and their function appears to regulate specific metabolic adjustments within each peripheral organ and to coordinate intertissue communication for homeostasis. Previous studies have established regulatory roles for several TF in fat and/or liver tissues in rodents and pigs, such as sterol regulatory element binding factor 1 and 2 (SREBF1 and SREBF2) (40), CCAAT/enhancer binding protein alpha (C/EBPα) (53), peroxisome proliferator-activated receptors beta (77), gamma (PPARγ) (76), and alpha (PPARα) (13, 39).

We hypothesize that transcriptional profiling of over 24,000 genes in key metabolic tissues of pigs, corroborated by blood metabolite analyses, will identify pathways and transcriptional networks of genes responding to fasting or MCR4 genotype. The results may implicate candidate genes to improve feed

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efficiency in pigs. We report 7,029 genes in adipose tissue and 1,831 genes in liver to be DE ($q \leq 0.05$) due to a 3-day fast. We also report on ensuing key biological processes. Major regulators of responding genes to fasting were identified by assessing the connection of DE TF to the DE genes. This study reports *1*) the first global study on feed deprivation using transcription profiling of key tissues in pig, *2*) identification of key TF in global fasting response, and *3*) analysis of shared biological pathways in the fasting response between fat and liver.

MATERIALS AND METHODS

Animals and Feed Treatments

Six-month-old Yorkshire gilts from a line selected for high feed efficiency, based on low residual feed intake (9) were used. Animals were fed ad libitum a crude protein standard swine diet with 20% crude protein until weaning (Kent Feeds, Muscatine, IA) and with 16–17.5% crude protein diet after weaning (Mid-State Milling, State Center, IA). Pigs were genotyped for the MC4R variant D298N (41), and 12 animals homozygous for N298 and 12 animals homozygous for D298 were randomly assigned to treatments. Starting at 06:00 on *day 1*, gilts were fed ad libitum with continuous access to feed until ~08:00 on *day 4* or had feed removed for 3 days in a randomized complete block design with a 2 × 2 factorial arrangement of treatments. A total of six animals were used for each combination of genotype and feed treatment across the four blocks that were defined as animals that underwent treatment on the same day. Each block contained at least one animal from each combination of genotype and feed treatment. All animals received water ad libitum. On *day 4* (09:00–11:30), pigs were killed by electric stunning, and samples of hepatic tissue and the 10th rib middle layer of backfat were rapidly collected, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. Pretreatment blood samples were collected the day before start of the feeding treatment from the jugular vein. Terminal blood samples were collected during postmortem exsanguinations. Blood samples were allowed to clot at 4°C overnight and centrifuged at 1,200 *g* for 30 min, and serum was collected and stored at −20°C. The protocol for animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of Iowa State University (protocol #12-04-5797-S).

Body Weight, Backfat, Liver Glycogen, and Blood Parameters

Pre- and posttreatment body weight and 10th rib backfat depth were measured on *days 0* and *4* (16:00–18:00). Backfat depth was measured by ultrasound (9) using an Aloka 500 V SSD ultrasound instrument fitted with a 3.5-MHz, 12.5-cm, linear-array transducer (Corometrics Medical Systems, Wallingford, CT). Liver glycogen was determined as reported previously (25). Briefly, samples of liver tissue (0.45 g) were extracted in cold perchloric acid (0.5 mol/l) using a Tissue Tearor homogenizer. Duplicate samples (300 μl) of each homogenate were then prepared for glycogen hydrolysis with 0.3 g/l amyloglucosidase (Sigma-Aldrich, St. Louis, MO) for 120 min at 38°C. The incubation was stopped by the addition of 0.6 mol/l perchloric acid and the samples clarified by centrifugation (1,500 *g*, 15 min at 4°C). Glucose (HK) assay kits (Sigma-Aldrich) were used to determine total micromolar glycosyl units (glucose, glucose-6-P, and glucose from glycogen) from the clarified samples and from the original homogenate (glucose, glucose-6-P only). Results were expressed as mg glycosyl per g wet tissue. Posttreatment serum samples were assayed for concentrations of glucose (hexokinase assay, Roche Diagnostics, Indianapolis, IN), urea (UREA/BUN kit by Roche Diagnostics), and triglyceride (TG, cat. #236-99; Diagnostics Chemicals, Oxford, CT). Nonesterified fatty acids (NEFA) concentration was measured on both pre- and posttreatment samples [HR Series-NEFA HR (2); Wako Diagnostics, Richmond, VA].

RNA Isolation and Microarray Hybridization

Total RNA was isolated and purified from liver and adipose tissues using the Qiagen RNeasy midi kit and the Qiagen RNeasy lipid tissue kit (Qiagen, Valencia, CA). Quality and quantity of RNA were determined by electrophoresis and spectrophotometry with the Agilent 2100 Bioanalyzer (Foster City, CA). Average RNA integrity numbers were 9.0 ± 0.5 for liver RNA and 8.2 ± 0.5 for fat RNA samples. Microarray target sample processing, target hybridization, washing, staining, and scanning steps were completed according to manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, 10 μg of total RNA from liver and adipose tissues was used to synthesize cDNA using a one-cycle cDNA synthesis kit. Resulting cDNA from each sample was used to transcribe biotinylated cRNA by T7 RNA polymerase and further fragmented and applied to the Affymetrix GeneChip Porcine Genome Array that contains 24,123 probe sets. Following hybridization at 45°C for 16 h, the array was washed and stained with streptavidin-phycoerythrin at an Affymetrix GeneChip Fluidics Station 450, and fluorescent signals were scanned using an Affymetrix GeneChip Scanner 3000 (3). Each tissue was assigned to one Fluidics station to remove potential station effects for within-tissue comparisons, and four modules within each station were intentionally confounded with the blocks.

Transcriptome

The Affymetrix GeneChip Porcine Genome Array probe set contains 11 paired perfect match (PM) and mismatch (MM) 25-mer probes (3). The probe-pair (PM-MM) data were used to determine the detection call (present call, marginal call, and absent call) by the modified Wilcoxon signed rank test of the MAS 5.0 software (50). For each tissue, probe sets with absent calls for all replicates in all treatments were removed, and the remaining probe sets were declared as expressed and established the transcriptome for that tissue.

Statistical Analyses

Animal performance, blood parameters, and liver glycogen. Body weight, average daily feed intake, backfat change, liver glycogen content, and blood parameters were analyzed by a mixed linear model with genotype, feed treatment, and their interactions as fixed effects and block as a random effect using the Mixed procedure of SAS/STAT software version 9.1.3 (SAS Institute, Cary, NC). The values for NEFA were natural log transformed to improve normality and homoscedasticity. Residual diagnostics identified no obvious concerns about the models used in all tests.

Gene expression. Affymetrix GeneChip Operating Software (GCOS) version 1.4.0 was used to obtain .CEL files at the GeneChip Facility at Iowa State University. Data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (series accession #GSE13528; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13528) and examined at the probe level with BioC version 2.0 (27) in R (60). All 24,123 probes, including absent calls, were analyzed. Three of 48 chips showed minor imaging artifacts. Probe-level measures were summarized into probe set level expression measures according to the MAS 5.0 algorithm (3) using the "affy" package (36) in R, except that the final step of aligning the trimmed means on the log scale was omitted because it was redundant with the median centering that was implemented in later steps. By replacing the PM and MM values with the corresponding probe set level measures, we constructed pseudoarray images, and visual data quality check indicated that previous imaging artifacts were successfully corrected at the probe set level. Base 2 logarithms were taken, and the median expression measures from each chip were examined for treatment effects by fitting an analysis of variance model with

genotype, feed treatment, their interaction, and block as fixed effects. Median centering normalization (81) was performed by subtracting the median expression from all expression measures on each chip on the logarithmic scale.

To determine the proper model for analysis of the expression data, 81 alternative mixed linear models were fitted to the normalized expression measures for each tissue and each gene separately using the mixed procedure in SAS/STAT software version 9.1.3 (SAS Institute). Models included MC4R genotype, feed treatment, and their interaction as fixed effects but differed from each other by including any subset of block (four levels), chip hybridization day (three levels), the process batch during washing of the chips (three levels), and Fluidics station final stage as recorded in the .XML files from GCOS (two levels), as either fixed or random effects. Variance components were estimated by the method of maximum likelihood, with nonnegativity boundary constraints. The model with block and hybridization day as random effects was selected based on gene-averaged SAS-reported information criteria, the gene-averaged standardized prediction sums of squares from leave-one-out cross-validation, specifics of the experimental design, and histograms of *P* values of contrasts of interest.

For the final model, variance components were reestimated with the method of residual maximum likelihood with nonnegativity boundary constraints. Fixed effect estimates and least squares means were obtained using generalized least squares, and fixed effects contrasts were tested using Wald-type F-tests with Kenward-Roger's correction (38). The positive false discovery control procedure (68) was used on each set of *P* values of contrasts of interest to compute *q* values. Genes with *q* values ≤ 0.05 were considered to be DE.

Affymetrix Probe Annotation

To obtain homologs and improved annotation, Affymetrix probe consensus sequences were used to BLAST against the well-curated NCBI's RefSeq database. Highest scores were used with a conservative cutoff of 1e-10 for the E-value. In total, 17,798 (73.8% of all) probes on the Affymetrix GeneChip Porcine Genome Array were assigned RefSeq annotation (Couture O, Callenberg K, Kaul N, Pandit S, Younes R, Hu Z, Dekkers J, Reecy J, Honavar V, Tuggle C, unpublished observations).

Pathway Analysis With Gene Ontology and KEGG

DE ($q \leq 0.05$) genes in fat and liver were divided into genes that were down- or upregulated in fat or liver as a result of fasting, creating four categories of genes. Using DAVID, an open access web-based functional annotation and clustering program (17), the four categories of genes were analyzed for overrepresented ($P \leq 0.05$) biological process categories based on Gene Ontology's (GO) Biological Process and Kyoto Encyclopedia Genes and Genomes (KEGG) databases. The *P* values for overrepresentation were computed by a modified Fisher's exact test, using the transcriptome for each tissue as background. The biological process categories were clustered using Functional Annotation Clustering (17), where the enrichment score for each cluster was computed as the negative log of the geometric mean of *P* values in the cluster. Additionally, the top 50 DE genes ($q \leq 0.05$) based on fold change in each of the four categories were functionally annotated to determine pathways that were populated by genes with high fold changes.

Pathway Studio Analyses

DE genes ($q \leq 0.05$) in fat or liver tissues were analyzed with Pathway Studio 5.0 (57), a text mining tool that detects relationships among genes, proteins, cell processes, and diseases as recorded in the PubMed database (Ariadne Genomics, Rockville, MD). The previously defined four categories of DE genes were assessed for reported (PubMed) associations with common regulatory TF and nuclear receptors based on regulation, direct regulation, binding, and/or promoter binding. Genes satisfying these criteria were counted for each regulatory TF. Because of the limited processing capacity of Pathway Studio, only DE genes with a fold change ≥ 2 were used in this analysis.

Real-time Quantitative PCR for Verification of DE Genes

Real-time quantitative PCR (qPCR) was used to verify fasting-induced differential expression of seven genes in adipose tissue and eight genes in liver. Total RNA was isolated from backfat of pigs that were arrayed by Affymetrix GeneChip Porcine Genome Array as described above and reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) (16). Real-time PCR was performed in duplicate using 100 ng cDNA (RNA equivalent) per 25 μl reaction or per well with the Brilliant kit (Stratagene, La Jolla, CA) on Bio-Rad MyiQ Single Color Real Time PCR Detection System (Bio-Rad). All probes and primers for real-time TaqMan PCR were designed (Supplementary Table 1[1]) using Primer Express 2.0 (Applied Biosystems, Foster City, CA), as previously described (16). The probes contained 3′Iowa Black FQ quencher and 5′ 6-FAM reporter (Integrated DNA Technologies, Coralville, IA). The PCR conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, then 4°C. Because variation in the expression of commonly used housekeeping genes such as GAPDH, HMBS, HPRT, SDHA, RPL32, YWHAZ, and UBC (75) was observed with a 3-day fasting treatment in one or both tissues, we normalized gene expression based upon the constant amount of RNA and cDNA amplified. This method has been proposed as the most reliable standardization of quantitative measurement of mRNA expression given that accurate estimation of total RNA is made with tools such as Agilent Bioanalyzer (8).

Quantification of gene expression was analyzed as previously reported (16). Briefly, cycle threshold (C_t) values averaged across duplicate readings were analyzed by a mixed linear model with genotype, feed treatment, and their interactions as fixed effects, and block as a random effect, using the Mixed procedure of SAS/STAT software version 9.1.3 (SAS Institute). A value of $P \leq 0.05$ was considered statistically significant. Fold change in expression was calculated as $2^{\Delta C_t}$ for each gene, where ΔC_t is the difference between least square mean C_t values for the fasting and ad libitum groups.

RESULTS

Fasting and MC4R Genotype Effects on Body Weight, Backfat, Liver Glycogen, and Serum Metabolites

The effects of a 3-day fast and MC4R genotype on body weight, backfat change, liver glycogen content, and serum metabolites are in Table 1. Fasted animals lost 5.5 kg or 6.7% of their body weight, whereas those fed ad libitum gained 2.5 kg or 3.2%. No evidence of a significant effect of MC4R genotype on body weight ($P = 0.75$) was noted. Pigs homozygous for N298 were previously reported (41) to have higher average daily feed intake ($P < 0.05$) than pigs homozygous for D298; however, we observed an opposite trend ($P = 0.09$) in our study.

Pretreatment backfat depth was not significantly different ($P = 0.17$) between feeding treatments but was significantly higher ($P = 0.05$) for pigs homozygous for N298 than for pigs homozygous for D298, which agrees with a previous report (41). Therefore, within-individual change in backfat depth due to fasting treatment was analyzed rather than posttreatment backfat depth to account for the initial backfat difference due to MC4R

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

Table 1. *Body weights, backfat changes, and serum parameters in pigs fed at ad libitum and fasted and that are homozygous for MC4R variant D298 or variant N298*

	Fed at Ad Libitum		Fasted			
Trait	D298	N298	D298	N298	SE	Feeding *P* Value
Feed intake, kg/day*	2.4[a]	2.2[a]	0	0	0.2	
Initial body weight, kg	81.0[a]	80.4[a]	82.0[a]	81.6[a]	1.8	0.4634
Final body weight, kg	83.5[a]	83.0[a]	76.5[b]	76.0[b]	2.2	0.0002
Initial backfat, mm	11.0[a]	13.2[b]	12.8[a,b]	13.2[b]	0.7	0.1738
Backfat [post-pre] change, mm	0.89[a]	0.64[a]	0.30[a]	−1.27[b]	0.5	0.0219
Glycogen in liver (ng/mg of liver)	14.2[a]	13.6[a]	4.2[b]	4.9[b]	2.0	<0.0001
Glucose, mg/dl	89.4[a]	78.0[a]	92.0[a]	90.7[a]	6.9	0.2444
Triglyceride, mg/dl	33.8[a]	29.0[a]	39.2[a]	40.2[a]	4.3	0.0708
Pretreatment NEFA ln(mEq/ml)†	4.7[a]	4.7[a]	4.9[a]	5.22[a]	0.2	0.1589
Posttreatment NEFA, ln(mEq/ml)	5.2[a]	5.4[a]	6.2[b]	6.0[b]	0.2	<0.0001
Urea, mg/dl	16.7[a,b]	17.1[a]	13.2[b,c]	12.5[c]	1.8	0.0056

Values are least square means and SE pooled across treatments, $n = 6$ per genotype by feeding treatment combination. The *P* values correspond to the main effect of feeding treatment. The effects of MC4R genotype and feed by genotype interaction were nonsignificant for all traits ($P \geq 0.09$) except for initial backfat depth ($P = 0.05$). Least square means within the same line that do not share the same superscript letter are significantly different at $P < 0.05$. *Feed intake was averaged over the 3-day treatment period and only pigs fed ad libitum were used to test the genotype effect. †Nonesterified fatty acid (NEFA) concentration data were natural log transformed.

genotype (Table 1). Fasted pigs had on average greater backfat loss than the fed group (ad libitum = 0.77 mm, fasted = −0.49 mm, $P = 0.022$), indicating that body lipid stores were mobilized as an energy source during feed deprivation.

Fasted pigs had ~67% less glycogen content compared with the fed group (ad libitum = 13.8 ng/mg, fasted = 4.6 ng/mg) (Table 1). Pretreatment NEFA concentration did not differ between the fasted and fed groups ($P = 0.16$); however, posttreatment serum NEFA concentrations were 127% greater ($P = 0.0002$) in fasted than ad libitum-fed animals. Posttreatment serum concentration of blood urea nitrogen was ~24% lower in fasted pigs ($P = 0.006$), but neither fasting nor MC4R genotype had a significant effect on posttreatment blood glucose or TG concentrations.

Fasting and MC4R Effects on Expression of Genes in Fat and Liver

Of 24,123 probe sets evaluated by microarray analysis, 19,885 and 19,162 provided data indicating the transcripts represented by these probe sets were expressed in fat and liver tissue, respectively. In response to a 3-day fasting treatment, 7,937 transcripts were identified to be DE ($q \leq 0.05$, $P \leq 0.029$) in fat, with 3,722 being upregulated and 4,215 downregulated. In liver, 1,832 transcripts were identified to be DE ($q \leq 0.05$, $P \leq 0.006$), of which 1,287 were upregulated and 545 downregulated (Supplementary Tables 2A and 2B). An improved annotation of probe sets on the Affymetrix GeneChip Porcine Genome Array (Couture O, Callenberg K, Kaul N, Pandit S, Younes R, Hu Z, Dekkers J, Reecy J, Honavar V, Tuggle C, unpublished observations) assigned gene names (BLASTN expectation score <1e-10) to 90 and 83% of DE transcripts ($q \leq 0.05$) due to fasting in liver and in fat, respectively. For simplicity, the changes in RNA levels detected by these annotated probe sets are referred to as gene expression differences for the rest of the paper. The gene-by-gene analysis that was conducted did not identify evidence of effects of MC4R genotype or of the interaction between genotype and feeding treatment at $q \leq 0.05$; thus, only the effects of fasting on gene expression were considered in further analyses.

Microarray Data Validation by qPCR

Expression patterns of seven genes representing the lipid biosynthetic pathways in subcutaneous adipose tissue and of eight genes in liver representing the gluconeogenesis, fatty acid oxidation, ketogenesis, or steroid synthesis pathways were verified by qPCR in fasted vs. ad libitum treatments (Fig. 1, Supplementary Table 3). For all tested genes, expression fold changes (ad libitum vs. fasted) were consistent in direction with the microarray results (Fig. 1). Statistical significance of fold changes (ad libitum vs. fasted) was also confirmed by qPCR ($P \leq 0.05$) for all genes except for insulin-like growth factor 1 (IGF1, $P = 0.13$) and IGF1 receptor (IGF1R, $P = 0.94$) in fat and for hydroxyacyl-coenzyme A dehydrogenase (HADHA, $P = 0.09$) in liver. IGF1 and IGF1R had low fold changes in microarray analysis (−1.35 and 1.84, respectively) and low expression levels in adipose tissue compared with the other four genes, whose absolute expression fold changes ranged from 2.5 to 27.4 in microarray analysis. Overall, the results obtained from microarray were statistically confirmed for 80% of the tested genes in liver and fat.

Biological Processes Affected by Fasting in Fat and Liver

To identify biological processes that respond to fasting, lists of DE genes in liver and fat were explored for overrepresentation of GO Biological Process categories, and the resulting overrepresented terms were clustered (Supplementary Table 4, A–D). In addition, the KEGG biological pathway database was used to identify specific biological pathways that were overrepresented by genes that responded to fasting ($P \leq 0.05$). A summary of KEGG annotations are shown in Fig. 2 and described further in the following sections.

Fasting Effects in Fat

Subcutaneous adipose tissue underwent significant gene expression changes that were reflected by the large number of DE genes (7,937), compared with liver (3,722). Indicative of a metabolic switch toward energy conservation in response to fasting, genes involved in direct energy-generating processes, such as oxidative phosphorylation, ATP synthesis, and TCA

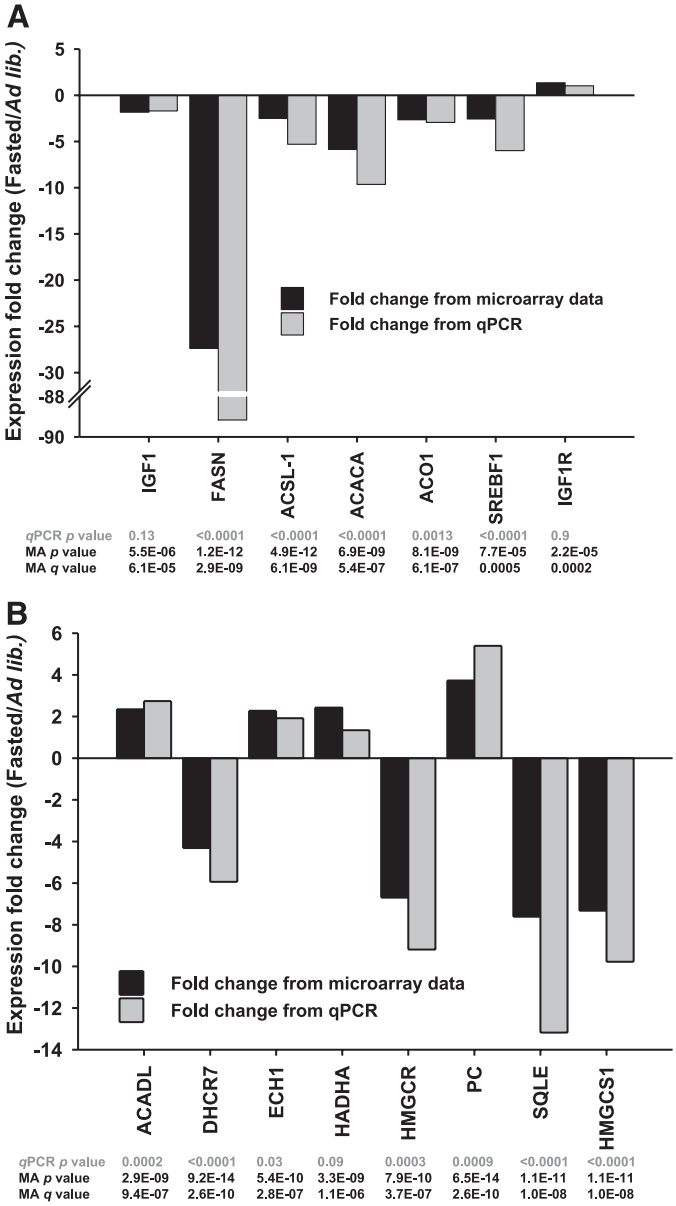

Fig. 1. Microarray data validation on a subset of differentially expressed genes ($q \leq 0.05$) by qPCR in liver and fat. *A*: representative genes in the lipid biosynthesis pathway in adipose tissue. *B*: representative genes in the gluconeogenesis, fatty acid oxidation, ketogenesis, or steroid synthesis pathways in liver. Official gene symbols were used as abbreviations. ACACA, acetyl-coenzyme A carboxylase alpha; ACSL-1, acyl-CoA synthetase long-chain family member 1; FASN, fatty acid synthase; ACO1, aconitase 1; SREBF1, sterol regulatory element binding factor 1; IGF1, insulin-like growth factor 1; HADHA, hydroxyacyl-Coenzyme A dehydrogenase; PC, pyruvate carboxylase; ACADL, acyl-Coenzyme A dehydrogenase, long chain; DHCR7, 7-dehydrocholesterol reductase; SQLE, squalene epoxidase; HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; ECH1, enoyl Coenzyme A hydratase 1; HMGCS1, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1. MA, microarray. Fold changes are expressed as (fasted/ad libitum), in which negative fold changes indicate downregulation while positive fold changes indicate upregulation of expression due to fasting.

cycle, were downregulated in fasted pigs. Likewise, genes involved in energy-intensive biosynthetic and catabolic processes were downregulated in the fasted pigs (Fig. 2, Supplementary Table 4A). This included genes encoding biosynthetic pathways, such as steroid and lipid biosynthesis, fatty acid synthesis, protein translation, amino acid synthesis, and ubiquinone biosynthesis, as well as genes encoding enzymes involved in catabolic processes, such as glucose metabolism, proteolysis, amino acid degradation, and pyruvate metabolism. Fasting induced upregulation of adipose genes involved in cytoskeleton reorganization, such as actin polymerization and cell-to-cell adhesion machinery, as well as genes involved in signal transduction pathways, such as adipocytokine signaling (Fig. 2, Supplementary Table 4B).

Fasting Effects in Liver

Liver is a primary site of glucose generation and sparing processes during fasting, such as gluconeogenesis and ketogenesis. Fasting induced upregulation of genes in gluconeogenesis as well as genes involved in energy derivation processes, such as fatty acid metabolism, glycolysis, pyruvate metabolism, proteasome, and amino acid degradation (Fig. 2, Supplementary Table 4C). Similar to what was observed for fat, liver downregulated expression of genes involved in lipid and steroid biosynthetic processes in response to fasting (Fig. 2, Supplementary Table 4D).

Genes and Pathways That Responded to Fasting in Both Fat and Liver

Genes found to be DE in both liver and adipose tissues (Fig. 3*A*) and KEGG pathways overrepresented ($P \leq 0.05$) in these genes were identified (Fig. 3*B*). In response to fasting, both liver and adipose tissues downregulated synthesis of steroids. When the top 50 significantly ($q \leq 0.05$) downregulated genes based on highest fold change in fat and in liver were analyzed, the steroid biosynthesis pathway was also the most significantly populated in both tissues ($P = 8.7e\text{-}05$ in fat, $P = 2.8e\text{-}10$ in liver) (Fig. 4). Within these top 50 downregulated liver genes were seven genes that encode enzymes involved in biosynthesis of steroids. These had fold changes ranging from 2.6 to 7.6 (3-hydroxy-3-methylglutaryl-coenzyme A reductase, 7-dehydrocholesterol reductase, sterol isomerase, isopentenyl-diphosphate δ isomerase 1, mevalonate decarboxylase, squalene epoxidase, and sterol-C5-desaturase-like). Similarly, the top 50 downregulated genes in fat contained four genes involved in steroid biosynthesis (mevalonate kinase, sterol isomerase, isopentenyl-diphosphate delta isomerase 1, and mevalonate decarboxylase), with fold changes ranging from 4.0 to 6.5.

Of 13 genes annotated by KEGG to encode enzymes in the steroid synthesis pathway, 12 are represented by probe sets on the Affymetrix GeneChip Porcine Genome Array. Ten of these genes, including the rate limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme reductase, were significantly downregulated ($q \leq 0.05$) in response to fasting in both fat and liver tissues. Exceptions were farnesyl diphosphate farnesyltransferase 1, which was significantly downregulated in liver but not in fat, and geranylgeranyl diphosphate synthase, which was downregulated in fat but not in liver (Table 3).

Pathways With an Opposite Fasting Response in Liver and Fat

Fasting induced five KEGG biological pathways in a distinct tissue-dependent manner, in which they were significantly downregulated ($P \leq 0.05$) in fat but upregulated in liver (Fig.

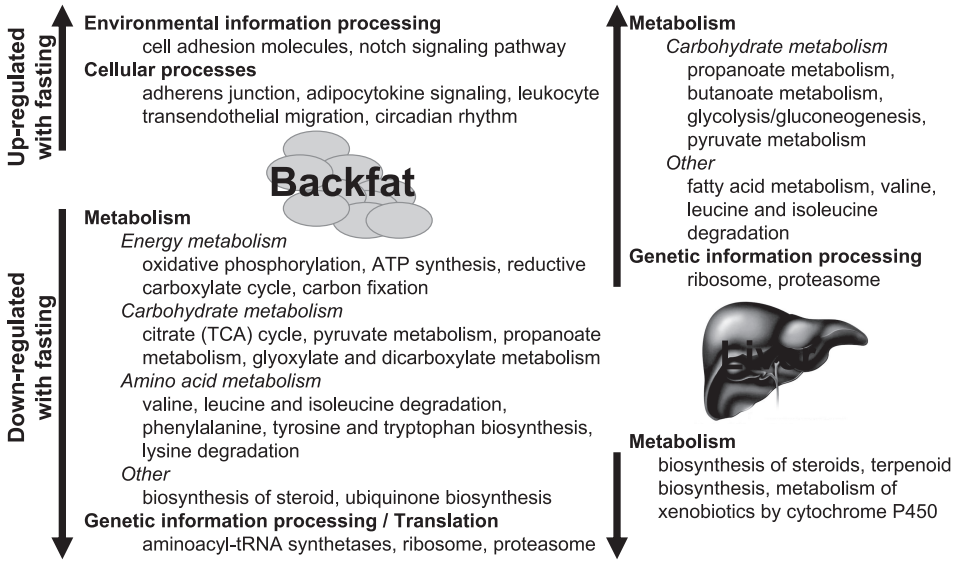

Fig. 2. Functional annotation of liver and adipose tissue-specific fasting response by identifying overrepresented KEGG biological pathways ($P \leq 0.05$) in differentially expressed genes. Biological processes were subdivided according to KEGG pathway maps.

3*B*). Two of these five pathways were glucose sparing (branched chain amino acids and propanoate metabolism) and were overrepresented among the 50 upregulated liver genes with the highest fold changes (Fig. 4). Genes in the propanoate metabolism and pyruvate metabolism pathways, which lead to succinyl CoA and acetyl CoA, respectively, were upregulated in liver and downregulated in fat. These provide starting and intermediate materials for the TCA cycle, indicating that fasting affected the TCA cycle in fat and liver in different fashions. Energy derived via the TCA cycle in liver likely was used for gluconeogenesis, which is energetically costly.

Key Transcriptional Regulators of Fasting Response

Most of the changes in RNA levels are likely due to changes in levels of transcription. To understand the main transcriptional regulation involved in the fasting response, genes that were up- and downregulated in liver and fat ($q \leq 0.05$) were analyzed for their connections to common TF or nuclear receptor regulators by using Pathway Studio 5.0. Connections between DE ($q \leq 0.05$) TF and their targets within the four DE gene lists were populated based on literature evidence of at least one of four interaction categories provided by Pathway Studio 5.0, which were promoter binding, binding, regulation, and direct regulation. Common regulators with the highest number of target genes across all four categories were determined (Fig. 5) and their target genes were functionally annotated (KEGG overrepresentation $P \leq 0.05$).

The most connected TF for downregulated genes in fat due to fasting was E2F transcription factor 1 (E2F1), which is known to regulate cell cycle and DNA replication. As expected, downregulated fat genes involved in cell cycle processes were overrepresented among the E2F1 targets (Fig. 5). In addition, several of the 10 most connected TF of downregulated fat genes are known to modulate homeostatic responses,

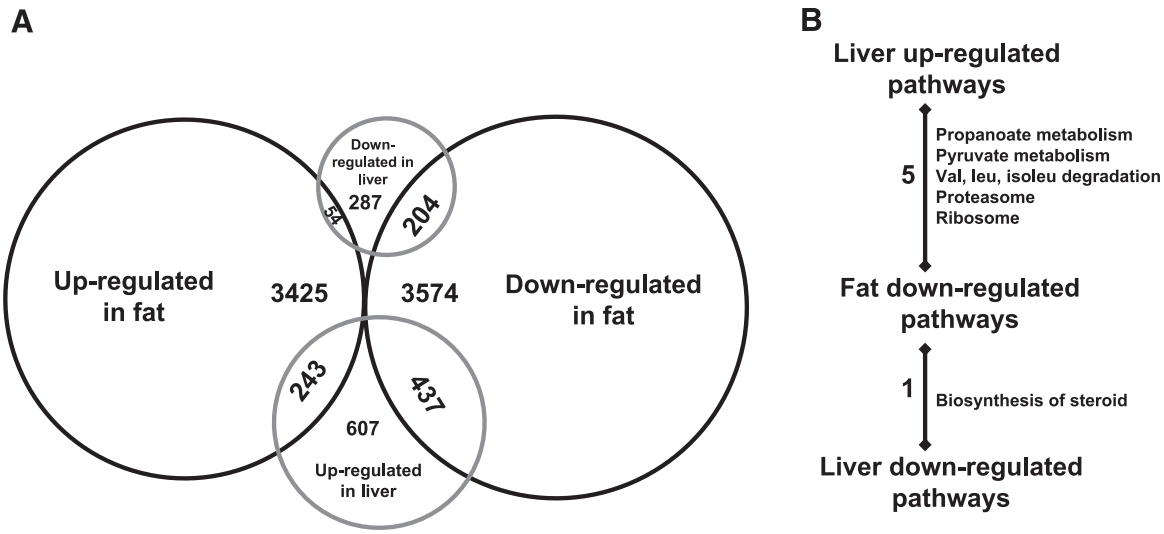

Fig. 3. Genes and pathways responding to fasting in liver and fat with either the same or opposite pattern in response to fasting. *A*: Venn diagram of overlapping up- and downregulated genes ($q \leq 0.05$) between liver and adipose tissue. *B*: shared up- and downregulated pathways and their functions (KEGG $P \leq 0.05$) in response to fasting across adipose and liver tissues.

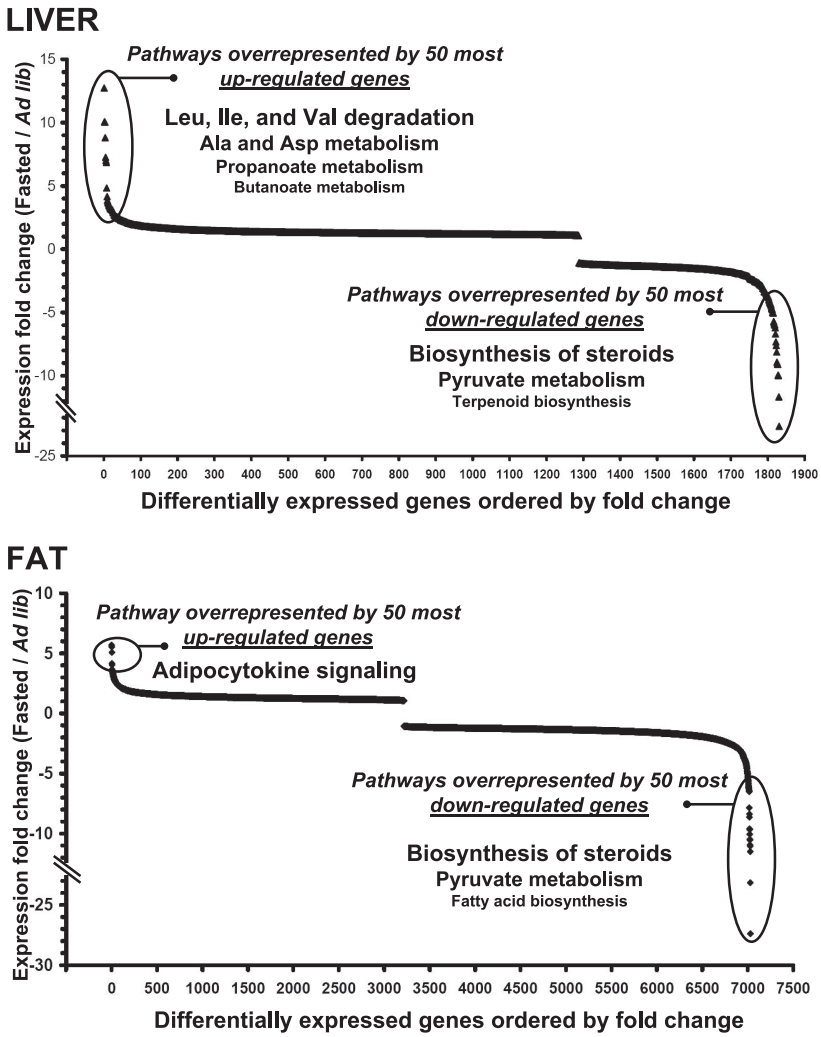

Fig. 4. Fold change distribution of differentially expressed genes due to fasting in fat and in liver. The functional annotation of the 50 most up- and downregulated genes based on fold change in fat and in liver (KEGG $P \leq 0.05$) shows that the biosynthesis of steroids pathway was most significantly overrepresented by the 50 most downregulated genes in both tissues. The highest, lowest, and the average fold changes were 5.7, 2.3, and 2.8 for the 50 most upregulated genes in fat; −3.96, −27.4, −6.61 for the 50 most downregulated genes in fat; 12.7, 2.02, and 3.3 for the 50 most upregulated genes in liver; −2.6, −22.7, −4.8 for the most 50 downregulated genes in liver.

including PPARγ (regulator of adipose differentiation and glucose metabolism), C/EBPα (adipogenesis, leptin expression), and SREBF1 (lipid and steroid synthesis). The most connected TF of downregulated liver genes due to fasting was tumor protein p53, whose target gene annotations were primarily in the cell cycle pathway, indicating decreased cell proliferation in response to fasting. Also, several of the 10 most prominent transcriptional regulators of downregulated liver genes are TF known to be involved in the fasting response; i.e., peroxisome proliferator activated receptor gamma coactivator-1 and SREBF2 (58, 65).

Among the four categories shown in Fig. 5, estrogen receptor 1 (ESR1) had the most target genes of all other identified common regulators of DE genes. Pathway analysis showed that adherens junction and focal adhesion pathways were overrepresented in the upregulated adipose target genes of ESR1, indicating that fasting induced ESR1-mediated adipocyte remodeling of the cytoskeleton and interaction with the extracellular matrix. Forkhead box O1A was also among the top 10 common regulators of upregulated genes in fat, and has been associated with metabolic adaptive response and in negative feedback signaling of insulin (62). The most connected TF of the upregulated genes in liver due to fasting was nuclear receptor co-repressor 1 (NCOR1), which is involved in repression of transcription by nuclear receptors (37). The target genes of NCOR1, however, did not result in significant overrepresentation of any KEGG category.

DISCUSSION

This is the first study to employ transcriptional profiling to investigate the global effects of fasting or MC4R genotype in the pig. Some reports are available on transcriptional profiling of fasting response using rodent models (49, 55), but the pig model more closely resembles human homeostatic mechanisms than rodents (54, 63). Our main findings demonstrate transcriptional responses across liver and adipose tissue with congruent changes in serum metabolites. Although changes in RNA expression do not always correspond to changes in protein expression or activity, we suggest that systemic changes in the expression of genes that belong to overrepresented GO or KEGG categories imply functional change.

Congruent to studies using rats (49, 55) we observed that, in response to fasting, fat and liver tissues downregulated energy-costly biosynthetic processes and upregulated genes involved in efficient energy utilization and conservation pathways, such as gluconeogenesis and β-oxidation of fatty acids in liver (41).

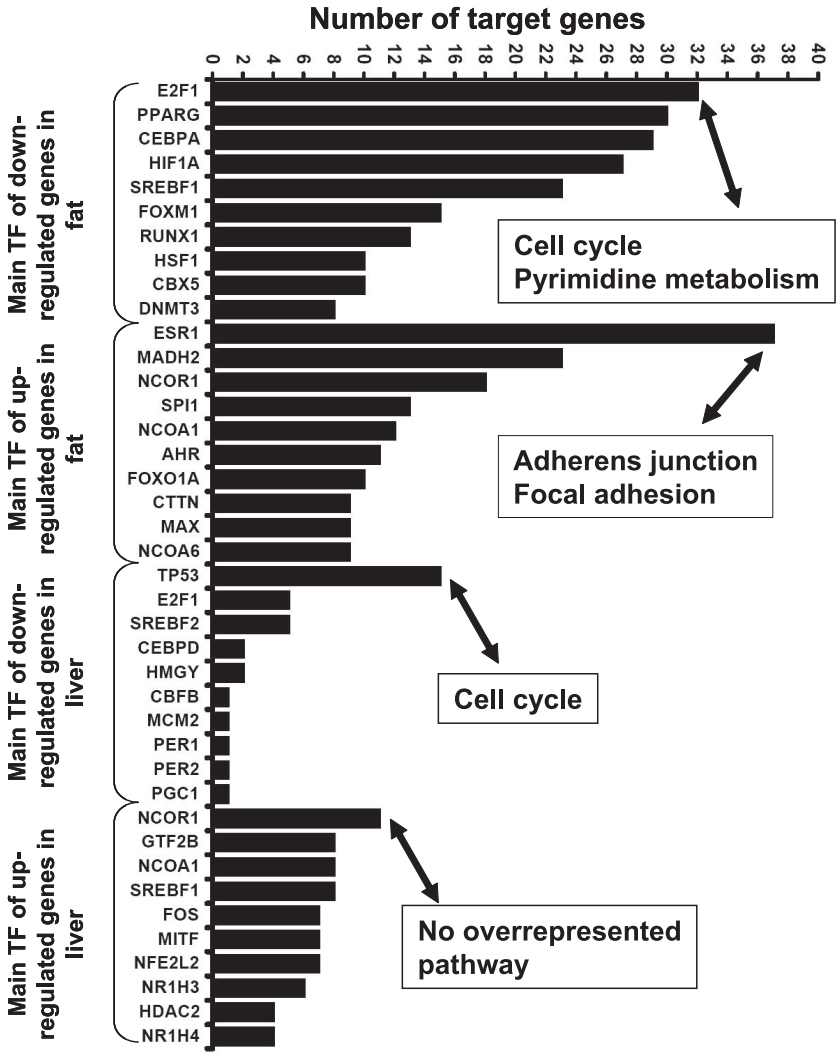

Fig. 5. Ten highest connected transcription factors (TF) for fasting response in the up- and downregulated genes in fat and liver and the pathways overrepresented (KEGG $P \leq 0.05$) by the target genes of the most connected TF in each list. Official gene symbols for the TF and nuclear receptors were used as abbreviations. CBFB, putative C module-binding factor; CBX5, chromobox homolog 5; CEBPA, CCAAT/enhancer binding protein alpha; CEBPD, CCAAT/enhancer binding protein delta; DNMT3, DNA (cytosine-5)-methyltransferase 3 beta; E2F1, E2F transcription factor 1; ESR1, estrogen receptor 1; FOS, FBJ murine osteosarcoma oncogene; FOXM1, forkhead box M1; GTF2B, general transcription factor IIB; HDAC2, histone deacetylase 2; HIF1A, hypoxia inducible factor 1, alpha subunit; HMGY, high mobility group AT-hook 1; HSF1, heat shock factor 1; MADH2, MAD homolog 2; MCM2, cell division control protein 21; MITF, microphthalmia-associated transcription factor; NCOA1, nuclear receptor coactivator 1; NCOR1, nuclear receptor co-repressor 1; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; NR1H3, nuclear receptor subfamily 1, group H, member 3; NR1H4, nuclear receptor subfamily 1, group H, member 4; PER1, period homolog 1; PER2, period 2; PPARG, peroxisome proliferator-activated receptor gamma; PGC1, peroxisome proliferator activated receptor gamma coactivator-1; RUNX1, runt-related transcription factor 1; SPI1, spleen focus forming virus proviral integration oncogene; SREBF1, sterol regulatory element binding factor 1; SREBF2, sterol regulatory element binding factor 1; TP53, tumor protein p53.

Fasting increased release of free fatty acid into circulation and decreased protein degradation, as indicated by decreased serum urea concentrations, consistent with findings in a separate study with a 3-day fast of pigs (67).

Contrary to a previous report (41), average ad libitum feed intake of pigs homozygous for allele D298 tended to be greater ($P = 0.09$) than intake of pigs homozygous for N298, although our results are based on a very small number and should, therefore, be interpreted with caution. In a preliminary study, feed intake of pigs from the same population as used in this study (9) did not change in response to intracerebroventricular injection of NDP-MSH, a potent activator of MC4R (C. R. Barb, unpublished data). In addition, a recent study demonstrated that cAMP accumulation did not differ between cells transfected with D298 and N298 variants (21), in contrast to the previous report on functional significance of D298N mutation (42). These results suggest that lack of evidence for MC4R effect on gene expression, body weight, backfat depth, and blood parameters in our study occurred perhaps due to lack of functional significance of D298N variant or the small sample size that was used in this study.

Fasting Induced Upregulation of Genes in Cell Adhesion and Angiogenesis Pathways in Fat

A novel finding in our study is that fasting induced upregulated expression of fat genes involved in the morphology and structure of adipocytes, as indicated by three main biological process clusters of cytoskeletal organization, vasculature development, and branching structure development being overrepresented among these genes (Supplementary Table 4B). Furthermore, three KEGG terms corresponding to adherens junction, cell adhesion molecules, and Notch signaling (Fig. 2) were also overrepresented among upregulated genes in fat. The fold changes, *q,* and *P* values of genes involved in fasting-induced angiogenesis and morphological changes are summarized in Table 2. No previous report has indicated that changes in cell-to-cell or cell-to-matrix pathways are associated with fasting. Interestingly, a recent whole genome SNP analysis in cattle has implicated similar pathways, such as cell adhesion and extracellular matrix, in controlling efficiency of feed utilization (6).

Metabolic response to feed deprivation has been divided into three phases according to changes in protein and lipid utiliza-

Table 2. *Statistical values of genes involved in the fasting induced morphological changes in the fat*

Pathways	Gene Description	GenBank Symbol	Affymetrix ID	*Fold Change	*q* Value	*P* Value
Angiogenesis	interleukin 8	IL8	Ssc.658	2.53	3.05E-02	1.54E-02
	insulin-like growth factor 2	IGF2	Ssc.9365	1.16	2.15E-02	9.75E-03
	natriuretic peptide receptor A/Guanylate cyclase A	NPR1	Ssc.24926	1.30	1.21E-03	2.55E-04
	angiogenin	ANG	Ssc.4679	1.93	7.08E-06	2.97E-07
	endothelial PAS domain protein 1	EPAS1	Ssc.8965	1.39	1.19E-02	4.62E-03
	vascular endothelial growth factor C	VEGFC	Ssc.12790	1.42	3.66E-02	1.95E-02
	laminin, alpha 5	LAMA5	Ssc.15173	1.46	3.39E-04	5.02E-05
	forkhead box C2	FOXC2	Ssc.19569	1.72	2.51E-02	1.19E-02
	angiogenic factor with G patch and FHA domains 1	AGGF1	Ssc.26953	1.14	4.05E-03	1.19E-03
	NMDA receptor regulated 1	NARG1	Ssc.12421	1.45	1.54E-03	3.44E-04
	angiopoietin-like 4	ANGPTL4	Ssc.8980	2.36	6.41E-04	1.14E-04
	B-cell translocation gene 1, antiproliferative	BTG1	Ssc.6833	1.19	3.94E-04	6.08E-05
	integrin, beta 3	ITGB3	Ssc.7556	1.26	1.79E-06	2.57E-05
	hairy and enhancer of split-related protein 2	HEY2	Ssc.24610	1.21	0.076992	0.05356
Adherens junction	mothers against DPP	SMAD2	Ssc.24402	1.28	3.82E-03	1.10E-03
	catenin (cadherin-associated protein)	CTNNB1	Ssc.14003	1.12	1.23E-03	2.60E-04
	protein tyrosine phosphatase, receptor type, F	PTPRF	Ssc.2337	1.26	8.98E-03	3.22E-03
	Wiskott-Aldrich syndrome-like	WASL	Ssc.21951	1.14	2.61E-04	3.63E-05
	epidermal growth factor receptor	EGFR	Ssc.55	1.73	8.08E-05	7.99E-06
	V-Yes-1 Yamaguchi sarcoma viral oncogene	YES1	Ssc.6801	1.27	1.78E-02	7.72E-03
	WAS protein family member 3	WASF3	Ssc.4177	1.29	2.87E-03	7.68E-04
	Partitioning defective 3 homolog	PARD3	Ssc.1382	1.57	4.38E-04	6.93E-05
	fibroblast growth factor receptor 1	FGFR1	Ssc.17773	1.41	1.95E-01	2.00E-01
	transcription factor 7-like 2	TCF7L2	Ssc.19409	1.41	9.77E-05	1.03E-05
Cell adhesion molecules	activated leukocyte cell adhesion molecule	ALCAM	Ssc.13385	1.61	2.21E-03	5.47E-04
	CD276 antigen	CD276	Ssc.2042	1.51	2.10E-04	2.74E-05
	CD34 antigen	CD34	Ssc.15747	1.20	2.02E-02	9.04E-03
	CD99 antigen	CD99	Ssc.3205	1.29	8.12E-04	1.54E-04
	claudin 3	CLDN3	Ssc.9102	1.53	3.22E-02	1.65E-02
	claudin 5	CLDN5	Ssc.4227	2.04	3.36E-04	4.98E-05
	huel-interacting protein	ESAM	Ssc.23336	1.44	4.48E-04	7.12E-05
	F11 receptor	F11R	Ssc.29060	1.20	4.37E-04	6.90E-05
	major histocompatibility complex, class I, A	HLA-A	Ssc.13780	1.52	9.76E-03	3.58E-03
	major histocompatibility complex, class II, DM alpha	HLA-DMA	Ssc.13777	1.14	4.03E-02	2.22E-02
	intercellular adhesion molecule 1 (CD54)	ICAM1	Ssc.11187	1.31	8.54E-03	3.03E-03
	integrin, beta 8	ITGB8	Ssc.23798	2.63	2.22E-03	5.53E-04
	junctional adhesion molecule 2	JAM2	Ssc.9217	1.49	2.14E-04	2.81E-05
	neural cell adhesion molecule 1	NCAM1	Ssc.6092	1.41	1.79E-02	7.76E-03
	platelet/endothelial cell adhesion molecule (CD31)	PECAM1	Ssc.14558	1.24	1.07E-02	4.01E-03
	protein tyrosine phosphatase, receptor type, F	PTPRF	Ssc.2337	1.26	8.98E-03	3.22E-03
	syndecan 2	SDC2	Ssc.2485	1.51	9.36E-07	1.54E-08
	selectin E	SELE	Ssc.16297	2.58	1.72E-02	7.41E-03
Notch signaling	presenilin 1	PSEN1	Ssc.12937	1.24	6.03E-04	1.05E-04
	numb homolog	NUMB	Ssc.20190	1.13	3.63E-03	1.03E-03
	E1A binding protein	EP300	Ssc.22177	1.13	3.24E-02	1.66E-02
	CREB binding protein	CREBBP	Ssc.17277	1.16	4.14E-03	1.22E-03
	SNW domain containing 1	SNW1	Ssc.17288	1.21	4.25E-02	2.39E-02
	Notch homolog 1, trasnlocation-associated	NOTCH1	Ssc.26169	1.32	5.34E-03	1.68E-03
	KIAA1528 protein	DTX2	Ssc.26800	1.31	3.07E-03	8.38E-04
	C-terminal binding protein 2	CTBP2	Ssc.27641	1.95	1.29E-06	2.77E-08
	notch homolog 2	NOTCH2	Ssc.19892	1.29	3.99E-04	6.18E-05
	mastermind-like 2	MAML2	Ssc.27051	1.37	4.10E-04	6.39E-05

*Fold changes are expressed as (fasted/ad libitum), in which positive fold changes indicate upregulation of expression due to fasting.

tion (15). Our study falls into phase I, which involves a period of adaptation resulting in glycogen breakdown and lipid mobilization, as indicated by our finding of increased NEFA release into the bloodstream and loss of 67% of liver glycogen content (Table 1). As free fatty acids are a major source of energy during fasting, capillaries embedded in subcutaneous adipose tissue likely require larger surface to ensure efficient transportation of free fatty acids. Angiogenic growth factors are released from tissues upon need for new blood vessel formation from preexisting vessels. In accordance with this, the expression of critical angiogenic genes, such as those encoding angiogenin (ANG), angiogenic factor with G patch and FHA domains 1 (AGGF1), and vascular endothelial growth factor (VEGF), were significantly upregulated in fat by fasting in our study. Several cytokines and growth factors have been investigated for their involvement in angiogenesis. The inflammatory cytokine, interleukin 8 (IL-8), has been shown to contribute to human melanoma progression by functioning as a mitogenic and angiogenic factor (61), and neutralizing antibodies of IL-8 inhibit angiogenesis and tumor growth (35). Fasting upregulated IL-8 expression 2.5-fold in the adipose tissue in the present study. Lee et al. (47) have investigated the angiogenic properties of IGF-II in an in vitro model and demonstrated that it directly induced angiogenesis by stimu-

lating migration and morphological differentiation of endothelial cells. Thus, our study indicates that a number of angiogenic factors, including VEGF, ANG, AGGF1, IL-8, and IGF-II, were significantly upregulated with fasting in the pig model.

Transcription factors endothelial PAS domain protein 1 (EPAS-1) and forkhead box C2 (FOXC2) have been identified as transcriptional regulators of angiogenic genes (72, 80). In response to fasting in pigs in our study, EPAS-1 and FOXC2 were significantly upregulated 1.4 and 1.7-fold, respectively. EPAS-1 is a basic helix-loop-helix/PAS domain TF that is expressed most abundantly in highly vascularized organs (79). EPAS-1 promotes angiogenesis via transactivation of VEGF and its receptor (72, 79), which agrees with our finding of fasting induced VEGF expression. FOXC2 was studied in a transgenic mouse model and increased expression of FOXC2 in adipose tissue promotes angiogenesis and vascular patterning (33, 80). FOXC2 directly targets integrin beta 3 (ITGB3), hairy and enhancer of split-related protein 2 (HEY2), and ANG2 (80) to promote angiogenesis; ITGB3 was upregulated 1.26-fold (q = 1.79E-06), while HEY2 had tendency to be upregulated 1.21-fold (q = 0.07) with fasting (Table 2).

Angiopoietin-like peptides (ANGPTL), such as ANGPTL 3, 4, and 6, have been shown to be involved in lipid, glucose, and energy metabolism independent of angiogenic effects (30). Of these ANGPTL, the Affymetrix array contained a transcript for only ANGPTL4, which was >2-fold upregulated in fasted pigs. Angiopoietin-like 4 has been shown to increase plasma TG levels via direct inhibition of lipoprotein lipase (51, 69) and may explain the tendency for increased levels (P = 0.07) of TG in the fasted group. The role of ANGPTL4 in angiogenesis is not clear and may have both pro- and antiangiogenic effects (11, 45). Although angiogenesis has been suggested to be an indicator for adipogenesis (10, 32), our data indicate decreased lipid biosynthesis (i.e., adipogenesis). Angiogenesis may have a negative relationship with adipogenesis during fasting-induced responses in growing animals, as the positive relationship between angiogenesis and adipogenesis has been established only during fetal fat mass development (10, 32). Taken together, fasting appears to induce expression of a number of genes in the pathway leading to angiogenesis.

Fasting Induced Upregulation of Genes Involves Cell-to-Cell and Cell-to-Matrix Communication in Fat

Successful vascular development depends on efficient cell-to-cell and cell-to-matrix communication and cytoskeletal reorganization (64). We found that genes such as cadherin-associated protein, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 1 (FGFR1) in the adherens junction, notch signaling, cell adhesion, and cytoskeletal organization pathways were significantly upregulated in fat of fasted pigs (Fig. 2, Supplementary Table 4B). Fasting induced expression of genes involved in morphological changes in adipose tissue, and these changes may have been partially mediated by ESR1, as many of its target genes were overrepresented in these pathways and consisted of genes such as EGFR, FGFR1, and E1A binding protein. Expression of ESR1 was increased 1.8-fold (Ssc.12290, q = 1.18E-05, P = 1.09E-04) in fat in response to fasting. No study has yet reported fasting-induced increases in ESR1 RNA levels in any tissue, although several metabolic syndromes, such as type II diabetes and atherosclerosis have been linked to polymorphisms of ESR1 (26, 34). Coupled with our finding, this suggests a potential role of ESR1 in homeostatic mechanisms that regulate fasting-induced changes of adipocyte cell-to-cell interactions.

Fasting-induced Lipolysis in Fat

Fat serves as the main TG storage site, which, in the face of feed deprivation, provides free fatty acids in the circulation as a source of energy by other tissues and for glycerol to be converted to glucose in liver. The greater concentration of circulating NEFA in fasted pigs and the significant decrease in backfat provide evidence for depletion of TG from the adipose tissue. While fasting induced depletion of TG, the expression of genes involved in synthesis of lipids decreased with fasting in both tissues and was verified by qPCR (Fig. 1).

Fasting-induced lipolysis in pigs differs from that in humans and in rodents and is not associated with increases in glucagon (66), epinephrine, or norepinphrine (56). Increased sensitivity of adipose tissue to β-adrenergic agonists during fasting in pigs has been noted (66), and our results suggest that the mechanism behind this may involve RNA level changes, as we found fasting to induce upregulation of the β-adrenergic receptor gene (Table 3). However, the expression of hormone-sensitive lipase (HSL) was not altered due to fasting (Table 3). This result may be species specific, as transcription of HSL was upregulated in adipose tissue after a 3-day fast in rats (71). Fasting-induced lipolysis in pigs may therefore be mediated via a previously described HSL-independent pathway (24). Paradoxically, TF FOXC2, which is known to increase sensitivity to cAMP/PKA-dependent signals and induce HSL expression (12), was increased 1.7-fold due to fasting (Table 3). Thus, the role of FOXC2 in mediating lipolysis may extend beyond regulation of HSL expression.

Liver Upregulated Gluconeogenesis and Fatty Acid Oxidation

The main goal of the fasting response is to maintain sufficient glucose concentration in the bloodstream, primarily through the liver, such that the nervous system has enough fuel. As with a previous fasting study in pigs (67), glucose concentration in the serum after 3 days of fasting was maintained at a similar concentration as that of ad libitum-fed pigs. This glucose homeostasis was likely mediated via gluconeogenesis and glycolysis pathways, because the genes encoding enzymes involved in these pathways, such as pyruvate carboxylase (PC), cytosolic and mitochondrial phosphoenolcarboxykinase 2, and the rate-limiting enzyme fructose-1,6-bisphosphatase (FBP1), were upregulated with fasting (Table 3). Although PC has not been implicated to be the rate-limiting step of hepatic gluconeogenesis (52), its 3.7-fold induction by fasting was higher than that of FBP1 (Table 3) and this was confirmed by qPCR (Fig. 1*B*). In addition, increased expression of genes involved in oxidation of fatty acids such as acyl-CoA dehydrogenase, enoyl CoA hydratase 1, and hydroxyacyl-CoA dehydrogenase, was observed in liver (Table 3) and these were confirmed by qPCR (Fig. 1*B*). The energy derived from fatty acid oxidation likely provided fuel for gluconeogenesis during fast. Furthermore, upregulation of branched amino acid degradation pathway genes in the liver suggests the accumulation of gluconeogenic and ketogenic precursors, utilizing amino acids derived from degradation of proteins by the proteasomal pathway. As adipose tissue does not

Table 3. *The statistical differential expression values of genes and pathways referred to in* RESULTS

Pathways	Gene Description	GenBank Symbol	Affymetrix ID	Tissue	*Fold Change	*q* Value	*P* Value
Gluconeogenesis	fructose-1,6-bisphosphatase	FBP1	Ssc.5127	liver	1.30	3.35E-04	3.96E-06
	phosphoenolcarboxykinase 2	PCK2	Ssc.19382	liver	1.44	8.70E-03	4.30E-04
	pyruvate carboxylase	PC	Ssc.17347	liver	3.73	2.56E-10	6.49E-14
Lipolysis	β-adenergic receptor gene	ADRB1	Ssc.16075	fat	1.30	4.00E-02	2.00E-03
	hormone sensitive lipase	HSL	Ssc.6784	fat	1.05	3.00E-01	4.30E-01
	forkhead box C2	FOXC2	Ssc.19569	fat	1.70	2.50E-02	1.20E-02
Adipogenesis	peroxisome proliferator-activated receptor gamma	PPARγ	Ssc.15274	fat	−1.32	2.39E-04	6.00E-03
	CCAAT/enhancer binding protein, alpha	C/EBPα	Ssc.15991	fat	−1.44	9.60E-03	3.50E-03
	sterol regulatory element binding factor 1	SREBF1	Ssc.15992	fat	−2.57	4.75E-04	7.69E-05
Fatty acid oxidation	acyl-CoA dehydrogenase, long chain	ACADL	Ssc.14530	liver	2.34	9.43E-07	2.79E-09
	enoyl CoA hydratase 1	ECH1	Ssc.5099	liver	2.27	2.83E-07	5.39E-10
	hydroxyacyl-CoA dehydrogenase	HADHA	Ssc.11580	liver	2.43	1.08E-06	3.31E-09
	acetyl CoA acyltransferase	ACAA1	Ssc.1225	liver	1.50	1.73E-05	9.59E-08
	carnitine palmitoyltransferase 1A	CPT1A	Ssc.8974	liver	1.64	7.70E-03	3.63E-04
	peroxisome proliferator-activated receptor alpha	PPARα	Ssc.9348	liver	1.40	5.80E-03	2.28E-04
Glucose metabolism	E2 component of pyruvate dehydrogenases	PDCE2	Ssc.14134	liver	1.10	1.80E-01	5.10E-02
	α-subunit of pyruvate dehydrogenase	PDHA1	Ssc.16248	liver	1.00	6.60E-01	9.60E-01
	β-subunit of pyruvate dehydrogenase	PDHB	Ssc.7604	liver	1.03	5.00E-01	4.50E-01
	pyruvate dehydrogenase kinase-4	PDK4	Ssc.1121	liver	1.04	5.90E-01	6.70E-01
Ketogenesis	3-hydroxymethyl-3-methylglutaryl-CoA lyase	HMGCL	Ssc.27317	liver	−1.30	1.50E-02	9.80E-04
	3-hydroxymethylglutaryl-CoA synthase 1	HMGCS1	Ssc.11126	liver	−7.33	1.02E-08	1.12E-11
	acetyl CoA acetyltransferase 2	ACAT2	Ssc.4426	liver	−9.10	1.94E-07	3.10E-10
Biosynthesis of steroid	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	Ssc.16088	fat	−1.54	8.10E-03	2.84E-03
	7-dehydrocholesterol reductase	DHCR7	Ssc.5455	fat	−3.45	3.50E-04	5.30E-05
	sterol isomerase	EBP	Ssc.5045	fat	−4.04	5.29E-06	1.99E-07
	isopentenyl-diphosphate delta isomerase 1	IDI1	Ssc.6714	fat	−5.60	1.01E-06	1.77E-08
	sterol-C5-desaturase	SC5DL	Ssc.15727	fat	−3.57	2.70E-07	2.12E-09
	mevalonate kinase	MVK	Ssc.3345	fat	−4.95	2.25E-04	3.01E-05
	mevalonate decarboxylase	MVD	Ssc.4685	fat	−6.50	4.43E-05	3.62E-06
	squalene epoxidase	SQLE	Ssc.8385	fat	−1.44	3.07E-02	1.55E-02
	geranyl geranyl diphosphate synthase	GGPS1	Ssc.14178	fat	−1.22	1.78E-03	4.15E-04
	farnesyl diphosphate farnesyltransferase 1	FDFT1	Ssc.6418	fat	1.09	1.61E-01	1.50E-01
	sterol regulatory element binding factor 2	SREBF2	Ssc.16976	fat	−1.23	3.40E-02	1.80E-02
	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	Ssc.16088	liver	−6.70	3.74E-07	7.88E-10
	7-dehydrocholesterol reductase	DHCR7	Ssc.5455	liver	−5.72	1.24E-08	1.45E-11
	sterol isomerase	EBP	Ssc.5045	liver	−3.69	2.56E-10	9.46E-14
	isopentenyl-diphosphate delta isomerase 1	IDI1	Ssc.6714	liver	−9.94	2.56E-10	3.20E-14
	sterol-C5-desaturase	SC5DL	Ssc.15727	liver	−3.36	4.26E-09	3.67E-12
	mevalonate kinase	MVK	Ssc.3345	liver	−1.74	9.89E-03	5.21E-04
	mevalonate decarboxylase	MVD	Ssc.4685	liver	−4.35	3.49E-04	4.17E-06
	squalene epoxidase	SQLE	Ssc.8385	liver	−7.61	1.02E-08	1.14E-11
	farnesyl diphosphate farnesyltransferase 1	FDFT1	Ssc.6418	liver	−1.71	8.28E-05	6.63E-07
	geranyl geranyl diphosphate synthase	GGPS1	Ssc.14178	liver	−1.11	4.14E-01	2.94E-01
	sterol regulatory element binding factor 2	SREBF2	Ssc.15889	liver	−2.70	3.70E-02	3.50E-03
Cell cycle regulation	E2F transcription factor 1	E2F1	Ssc.1859	liver	−1.95	4.17E-03	1.43E-04
	CCAAT/enhancer binding protein, delta	C/EBPδ	Ssc.10025	liver	−1.47	1.55E-02	1.01E-03
	tumor protein p53	TP53	Ssc.15917	liver	−1.31	4.13E-02	4.29E-03
	cyclin-dependent kinase 2	CDK2	Ssc.27616	liver	−1.24	4.60E-02	5.00E-03
	cyclin E1	CCNE1	Ssc.18187	liver	−1.43	4.00E-02	4.00E-03
	E2F transcription factor 1	E2F1	Ssc.1859	fat	−2.09	1.18E-05	5.90E-07
	cyclin-dependent kinase 2	CDK2	Ssc.27616	fat	−1.27	1.89E-03	4.50E-04
	cyclin E1	CCNE1	Ssc.18187	fat	−1.34	6.01E-03	1.96E-03

*Fold changes are expressed as (fasted/ad libitum), in which negative fold changes indicate downregulation, while positive fold changes indicate upregulation of expression due to fasting.

serve as the primary site of glucose sparing during fasting, these pathways were downregulated in fat.

A key transcriptional regulator of fatty acid oxidation, PPARα, has previously been implicated in the fasting adaptation of liver in pigs (14). Congruent with fasting-induced activation of fatty acid oxidation in the liver, PPARα was upregulated 1.4-fold in our fasted animals. Also, degradation of amino acids and pyruvate for gluconeogenesis was evidenced by upregulation of genes encoding enzymes in the TCA cycle, such as succinate dehydrogenase complex subunits B and D and isocitrate dehydrogenases 1 and 3. During fasting in pigs, the TCA cycle is likely facilitated by acetyl CoA derived mainly from amino acids and glycerol precursors, rather than from glucose, because the expression of genes encoding the pyruvate dehydrogenase complex components, such as E2 component, α- and β-subunits, and pyruvate dehydrogenase kinase-4, was not altered (Table 3).

Fasting Effects on Ketogenesis and Hepatic Cell Cycling

With feed deprivation, the increased demand for β-oxidation of fatty acids in the liver results in ketogenesis. However, in

our study, genes encoding enzymes involved in ketogenesis, such as acetyl CoA actyltransferase 2 and 3-hydroxymethyl-glutaryl-CoA synthase 1 (HMGCS1), were downregulated (Table 3), and the expression was verified for HMGCS1 by qPCR (Fig. 1*B*). In addition, the serum concentrations of 3-hydroxybutyrate ($P = 0.40$) and acetate ($P = 0.26$) were not significantly altered after fasting (data not shown). Depletion of only about 67% of liver glycogen content was observed in our fasted pigs (Table 1), compared with rodents that show complete liver glycogen depletion after 3-day fasting. This partly explains the lack of evidence for ketogenesis because glucose derived from glycogenolysis and gluconeogenesis during the 3-day fast may have been sufficient to fuel the nervous system. Differences between rats and pigs in ketone body generation in response to feed deprivation have been reported previously (1), and have been attributed to the low rate of β-oxidation in pigs compared with rodents.

During fasting, rat hepatocytes undergo decreased cell proliferation (43), which is consistent with our finding that liver genes involved in cell cycling were significantly downregulated. Common regulators of downregulated liver genes due to fasting included tumor protein 53, E2F1, and C/EBPδ, and their target genes were involved in cell cycle or cell proliferation, such as cyclin-dependent kinase 2 and cyclin E1. This indicates that a decrease in proliferation of the hepatocytes to preserve energy during fasting likely occurred in pigs, as previously seen in rats.

Fasting Downregulated Steroid Biosynthesis in Both Liver and Fat

In rats, hepatic biosynthesis of steroids was decreased 10-fold after 48-h fasting (19), and expression of genes encoding enzymes involved in the steroid biosynthesis pathway was also significantly downregulated (55). In pigs, a tissue-level decrease in cholesterol due to fasting, combined with an increased serum cholesterol concentration, has been reported (22). Our finding of downregulation of 12 genes encoding enzymes involved in steroid biosynthesis, in part, explains these results. The protein SREBF2, which stimulates transcription of genes that encode enzymes involved in cholesterol synthesis (20), was downregulated 2.7-fold in liver and 1.23-fold in fat in response to fasting in our study (Table 3). SREBF2 was also one of the 10 TF with the greatest connectivity to the downregulated liver genes and the 18th most connected among TF of downregulated fat genes, emphasizing the importance of SREBF2 and steroid synthesis in the adaptive response to fasting.

Conclusions

A series of genes and biological pathways that respond to fasting in pigs was identified. A fasting-induced switch to a conservation mode of energy utilization by downregulating costly lipid, steroid, and protein biosynthetic processes was established. Fasting upregulated genes involved in angiogenesis and cell-to-cell signaling in fat and upregulated pathways involved with efficient energy utilization and conservation in liver, such as gluconeogenesis and β-oxidation of fatty acids. Our study implicates involvement of several known and several new TF in the adaptive fasting response in pigs, including PPARγ, C/EBPα, SREBF1, and EPAS-1, FOXC2, and ESR1. Our results support that during the early fasting stage, lipid stores are predominantly utilized and fat undergoes morphological changes that promote angiogenesis. Enhanced angiogenesis likely provides efficient lipid mobilization into the bloodstream and such morphological changes that target cell-to-cell and cell-to-matrix interactions are, in part, transcriptionally controlled by ESR1.

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