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# Transcript profiles in longissimus dorsi muscle and subcutaneous adipose tissue: A comparison of pigs with different postweaning growth rates

## Abstract

Although most pigs recover rapidly from stresses associated with the transition of weaning, a portion of the population lags behind their contemporaries in growth performance. The underlying biological and molecular mechanisms involved in postweaning differences in growth performance are poorly understood. The objective of this experiment was to use transcriptional profiling of skeletal muscle and adipose tissue to develop a better understanding of the metabolic basis for poor weaned-pig transition. A total of 1,054 pigs was reared in commercial conditions and weighed at birth, weaning, and 3 wk postweaning. Transition ADG (tADG) was calculated as the ADG for the 3-wk period postweaning. Nine pigs from both the lowest 10th percentile (low tADG) and the 60th to 70th percentile (high tADG) were harvested at 3 wk postweaning. Differential expression analysis was conducted in longissimus dorsi muscle (LM) and subcutaneous adipose tissue using RNA-Seq methodology. In LM, 768 transcripts were differentially expressed (DE), 327 with higher expression in low tADG and 441 with higher expression in high tADG pigs ( $q < 0.10$ ). Expression patterns measured in LM by RNA-Seq were verified in 30 of 32 transcripts using quantitative PCR. No DE transcripts were identified in adipose tissue. To identify biological functions potentially underlying the effects of tADG on skeletal muscle metabolism and physiology, functional annotation analysis of the DE transcripts was conducted using DAVID and Pathway Studio analytic tools. The group of DE genes with lower expression in LM of low tADG pigs was enriched in genes with functions related to muscle contraction, glucose metabolism, cytoskeleton organization, muscle development, and response to hormone stimulus (enrichment score  $> 1.3$ ). The list of DE genes with higher expression in low tADG LM was enriched in genes with functions related to protein catabolism (enrichment score  $> 1.3$ ). Analysis of known gene-gene interactions identified possible regulators of these differences in gene expression in LM of high and low tADG pigs; these include forkhead box O1 (FOXO1), growth hormone (GH1), and the glucocorticoid receptor (NR3C1). Differences in gene expression between poor transitioning pigs and their contemporaries indicate a shift to decreased protein synthesis, increased protein degradation, and reduced glucose metabolism in the LM of low tADG pigs.

## Disciplines

Agriculture | Animal Sciences | Genomics | Other Nutrition

## Comments

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# Transcript profiles in longissimus dorsi muscle and subcutaneous adipose tissue: A comparison of pigs with different postweaning growth rates<sup>1</sup>

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**ABSTRACT:** Although most pigs recover rapidly from stresses associated with the transition of weaning, a portion of the population lags behind their contemporaries in growth performance. The underlying biological and molecular mechanisms involved in postweaning differences in growth performance are poorly understood. The objective of this experiment was to use transcriptional profiling of skeletal muscle and adipose tissue to develop a better understanding of the metabolic basis for poor weaned-pig transition. A total of 1,054 pigs was reared in commercial conditions and weighed at birth, weaning, and 3 wk postweaning. Transition ADG (tADG) was calculated as the ADG for the 3-wk period postweaning. Nine pigs from both the lowest 10th percentile (low tADG) and the 60th to 70th percentile (high tADG) were harvested at 3 wk postweaning. Differential expression analysis was conducted in longissimus dorsi muscle (LM) and subcutaneous adipose tissue using RNA-Seq methodology. In LM, 768 transcripts were differentially expressed (DE), 327 with higher expression in low tADG and 441 with higher expression in high tADG pigs ( $q < 0.10$ ). Expression patterns measured in LM by RNA-Seq were verified in 30 of 32

transcripts using quantitative PCR. No DE transcripts were identified in adipose tissue. To identify biological functions potentially underlying the effects of tADG on skeletal muscle metabolism and physiology, functional annotation analysis of the DE transcripts was conducted using DAVID and Pathway Studio analytic tools. The group of DE genes with lower expression in LM of low tADG pigs was enriched in genes with functions related to muscle contraction, glucose metabolism, cytoskeleton organization, muscle development, and response to hormone stimulus (enrichment score  $> 1.3$ ). The list of DE genes with higher expression in low tADG LM was enriched in genes with functions related to protein catabolism (enrichment score  $> 1.3$ ). Analysis of known gene-gene interactions identified possible regulators of these differences in gene expression in LM of high and low tADG pigs; these include forkhead box O1 (FOXO1), growth hormone (GH1), and the glucocorticoid receptor (NR3C1). Differences in gene expression between poor transitioning pigs and their contemporaries indicate a shift to decreased protein synthesis, increased protein degradation, and reduced glucose metabolism in the LM of low tADG pigs.

**Key words:** pig, postweaning growth, transcriptional profiling

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## INTRODUCTION

The transition at weaning is one of the most stressful events in the pig's life and is associated with poor feed intake, reduced growth performance, and impaired gastrointestinal function (Moeser et al., 2007; Wijtten et al., 2011). Improvements in technology related to nutrition, health, and management have been utilized to minimize the adverse effects of weaning stress; however, there is still a tremen-

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dous amount of within-population variation in growth performance during the postweaning transition period. Previous research indicates that poor performance during the period immediately postweaning is associated with poor overall wean-to-finish performance and increased mortality (Jones, 2012; Jones et al., 2014). Poor energy intake and decreased protein and lipid deposition rates have been identified as key factors associated with low ADG during the postweaning transition period (Jones et al., 2012; Jones and Patience, 2014). Although numerous contributing factors have been identified, many of the underlying biological and molecular mechanisms involved in postweaning differences in growth performance are poorly understood.

Skeletal muscle and adipose tissue are key components of growth and are capable of physiological adaptations to a variety of conditions, including disease, stress, and reduced nutrient availability. High-throughput sequencing techniques, such as RNA-Seq, are used to measure the expression of the transcriptome of a tissue and may help to identify complex biological mechanisms that are involved in variable postweaning outcomes. Analyzing RNA profiles at critical points postweaning may enable us to identify molecular pathways that could be targeted to increase productive efficiency by optimizing genetics, management, and nutrition at the farm level. The objective of this study was to investigate the molecular basis for poor weaning transition in pigs reared in commercial conditions using RNA-Seq transcriptional profiling of muscle and adipose tissue.

## MATERIALS AND METHODS

All experimental procedures adhered to the ethical and humane use of animals for research and were approved by the Iowa State University Institutional Animal Care and Use Committee (number 2-11-7095-S).

### *Animals and Housing*

Over a 3.5-d period 1,500 pigs (Danbred 600 × Newsham NC32) were farrowed at a commercial farm. Before suckling, pigs were weighed individually to obtain birth weights and were tagged with a unique identification number. All procedures from birth until weaning were performed according to normal procedures at the source farm, including cross-fostering among litters to standardize litter size among sows. At 16 or 17 d of age, a total of 1,054 pigs was randomly selected from this farrowing group, weaned, and transported to a commercial wean-to-finish barn. Pigs were sorted by sex and randomly allotted to 40 pens with 26 to 27 pigs per pen.

Pigs were weighed individually at 0 and 3 wk postweaning. Transition ADG (**tADG**) was calculated as the ADG between wk 0 and 3 postweaning. All pigs were fed the same diets in the phase-feeding program utilized by the commercial producer. Pigs originated from a sow herd that was negative for porcine reproductive and respiratory syndrome virus (**PRRSv**). However, PRRSv and influenza A virus outbreaks were confirmed in wk 2 postweaning in the wean-to-finish barn where the study was conducted.

### *Tissue Collection and Sample Preparation*

Nine pigs from both the lowest 10th percentile (low tADG) and the 60th to 70th percentile (high tADG) for tADG were used to create 9 pairs of pigs, with pairs derived from litters of the same size and from sows of the same parity. These 9 pairs (18 total pigs) were transported approximately 45 min to the Iowa State University Diagnostic Laboratory, where they were euthanized via captive-bolt stunning followed by exsanguination. Samples of subcutaneous back fat between the eighth and last ribs and longissimus dorsi muscle (**LM**) were rapidly collected, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for later isolation of RNA. The remainder of each whole carcass, including blood, was stored at  $-20^{\circ}\text{C}$ . Carcasses were later ground, homogenized, and subsampled. Subsamples were freeze-dried and ground through a 1-mm screen and were analyzed for DM, ash, crude fat, and nitrogen content. Dry matter of feed and fecal samples was determined by drying samples in an oven at  $105^{\circ}\text{C}$  to a constant weight. Ash was determined by method 942.05 (AOAC International, 2005). Crude fat was determined by ether extraction according to method 920.39 (AOAC International, 2005). Nitrogen content was determined by the Kjeldahl method according to method 981.13 (AOAC International, 2005). Calibration of the N assay was conducted with a glycine standard (N content 18.7%). Upon analysis, N content of the glycine standard was determined to be  $18.7\% \pm 0.07\%$ . Crude protein was expressed as nitrogen content  $\times 6.25$ . All chemical analyses were performed in duplicate and were repeated when the intraduplicate coefficient of variation exceeded 1%. On the basis of the subsample chemical composition, total body composition was calculated for water, protein, lipid, and ash using empty BW at harvest.

Total RNA was isolated and purified from LM and adipose tissues using Ambion MagMAX total RNA isolation kits (Life Technologies, Carlsbad, CA) per the manufacturer's instructions. Quality and quantity of RNA were determined by using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Mean RNA integrity numbers were

$8.7 \pm 0.60$  for LM RNA samples and  $7.4 \pm 0.61$  for adipose tissue RNA samples.

The RNA sequencing libraries were prepared using TruSeq RNA Sample Prep Kits (Illumina Inc., San Diego, CA), and samples were sequenced by  $2 \times 100$  bp paired-end sequencing on an Illumina HiSeq 2000 instrument at the Iowa State DNA facility. All RNA samples were indexed, and 10 samples were loaded per flow cell lane. High and low tADG samples for each tissue were distributed randomly with roughly equal numbers of each tissue, and high and low groups were distributed within a flow cell so that tissue and group were not confounded in a flow cell lane.

### RNA-Seq Analysis

Raw sequence data were transferred to the Iowa State University Genome Informatics Facility. The raw read sequences were aligned to the *Sus scrofa* genome build 10.2 (Ensembl release 70) using the Genomic Short-read Nucleotide Alignment Program (GSNAP; version 2012-07-20; Wu and Nacu, 2010). Reads that mapped uniquely to the genome were utilized for downstream analysis and differential expression. For each gene, raw read counts were then determined using HTSeq and the Ensembl gene set GTF annotation file (version 70; available at <ftp://ftp.ensembl.org/pub/>). All sequence data are publicly available at the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) under GEO data set ID GSE65983.

### Statistical Analyses

**Growth and Body Composition.** Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with the variables tADG (high vs. low) and parity (1 vs. 2+) as fixed effects. Differences between least squares means were separated using the PDIF option of SAS, with results considered significant if  $P < 0.05$  and trends if  $P > 0.05$  and  $< 0.10$ .

**Gene Expression.** Statistical analysis of RNA-Seq read counts were performed using the QuasiSeq package in R (Lund et al., 2012). The raw read counts were normalized using upper quartile normalization. Normalized read counts were fit as the dependent variable in a generalized linear model, assuming a negative binomial distribution, including the variables tADG (high vs. low), parity (1 vs. 2+), and birth weight (in kg) as fixed effects. This model was compared with a reduced model, including all fixed effects except tADG to test for differences in gene expression as a function of tADG. The QuasiSeq software allows users to account for overdispersion in count data by modeling the estimated overdispersion by the total

number of read counts in a gene. A spline correction was used to account for some of the effects of overdispersion. The q-value method was used to correct for multiple testing as a means to control the false discovery rate (FDR; Storey and Tibshirani, 2003). Genes were considered to be differentially expressed (DE) if  $q < 0.10$ . There were no DE genes ( $q < 0.10$ ) identified in adipose tissue samples; therefore, additional analyses were conducted on only LM sample genes.

**Pathway Analysis with Gene Ontology and Kyoto Encyclopedia Genes and Genomes.** Genes in LM that were DE were divided into 2 gene lists: 1) higher expression in high tADG pigs and 2) higher expression in low tADG pigs. Using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7, an open-access Web-based functional annotation and clustering program (Huang et al., 2009a,b), the DE gene lists were analyzed for overrepresented biological process and metabolic function categories based on Gene Ontology (GO) or Kyoto Encyclopedia Genes and Genomes (KEGG) pathway categories. The *P*-values for overrepresentation of GO terms and KEGG pathways were computed by a modified Fisher's exact test, using the LM transcriptome as background. The Benjamini-Hochberg method was used to correct for multiple testing as a means to control the FDR. The individual GO terms and KEGG pathways were considered significant if  $FDR < 0.10$ . The GO biological process terms were clustered using the functional annotation clustering tool, where the enrichment score for each cluster was computed as the negative log of the geometric mean of *P*-values in the cluster. The resulting statistically significant clusters (enrichment score  $> 1.3$ ) were further curated to keep only GO terms with *P*-values  $< 0.05$ .

**Pathway Studio Analysis.** Genes in LM that were DE were analyzed with Pathway Studio version 9.0 (Nikitin et al., 2003), 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 data sets were studied using subnetwork enrichment analysis to find statistically significant entities connecting DE genes with higher expression in high or low tADG pigs. Common gene hubs were obtained on the basis of reported (PubMed) interactions, which were defined as regulation, direct regulation, promoter binding, and/or expression.

### Quantitative Reverse Transcriptase PCR for Verification of DE Genes

Quantitative reverse transcriptase PCR (qRT-PCR) was used to verify DE of 32 genes in LM. The 32 genes were selected to represent key pathways and biological functions identified by DAVID and Pathway Studio

analytical tools. Total RNA was isolated from LM of all 18 pigs using Norgen Animal Tissue RNA purification kits (Norgen Biotek Corp., Thorold, ON, Canada) and reverse transcribed to cDNA using SuperScript VILO cDNA synthesis kits (Life Technologies). For the qRT-PCR, DELTAgene Assays (Fluidigm, San Francisco, CA) were used (Supplementary Table 1), and specific sequences were obtained after 12 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 4 min using 2x TaqMan PreAmp Master Mix (Life Technologies). The preamplified products were 10-fold diluted and further amplified with the 2x SsoFast EvaGreen Supermix with Low ROX (Biorad, Hercules, CA) in a 48.48 Dynamic Array on a Biomark system (Fluidigm). The relative expression value of each gene was calculated relative to the housekeeping gene topoisomerase II beta (*TOP2B*; Erkens et al., 2006) using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). Data were analyzed using the PROC MIXED procedure of SAS with the same model as the RNA-Seq data as described above. Differences between least squares means were estimated using the PDIF option of SAS. To control for multiple testing, FDR (Benjamini and Hochberg, 1995) was estimated. Results were considered significant if  $FDR < 0.10$ .

## RESULTS

### Growth and Body Composition

High tADG pigs gained 130% more BW than low tADG pigs (220 vs. 96 g/d;  $P < 0.0001$ ; Table 1) during the 3-wk postweaning transition period. The mean tADG from the population of 1,054 pigs from which pigs for the experiment were selected was 197 g/d (Jones et al., 2014). Birth weight, weaning weight, and ADG from birth to weaning were similar ( $P > 0.10$ ) between high and low tADG pigs. Low tADG pigs were higher in whole-body water ( $P < 0.05$ ), tended to have greater ash percentage ( $P = 0.06$ ), and were lower in body lipid content ( $P < 0.01$ ).

### Read Alignment Statistics

For LM, an average of 15.3 million paired-end reads was generated per sample (Supplementary Table 2). For adipose tissue, an average of 16.4 million paired-end reads was generated per sample. In total, 82% of LM and 83% of adipose tissue reads were uniquely mapped back to the reference genome after alignment. There were 15,569 and 17,802 expressed genes in LM and adipose tissue, respectively, that mapped to the *Sus scrofa* genome. Additional details on read mapping and counting statistics are available in Supplementary Table 2.

**Table 1.** Body weight, ADG, and body composition of low and high transition ADG (tADG) pigs

Item	Low tADG	High tADG	SEM	P-value
<i>n</i>	9	9	—	—
BW, kg				
Birth	1.26	1.44	0.103	0.2362
Weaning	4.51	5.23	0.350	0.1641
3 wk postweaning	6.23	9.19	0.383	<0.0001
ADG, g/d				
Birth to weaning	197	230	17.3	0.1972
0–3 wk postweaning	96	220	5.0	<0.0001
Body composition, <sup>1</sup> %				
Water	68.5	66.2	0.51	0.0055
Protein	15.9	15.9	0.14	0.9900
Lipid	12.6	15.5	0.52	0.0012
Ash	3.0	2.4	0.19	0.0571

<sup>1</sup>Measured at 3-wk postweaning.

### Detection of DE Genes

RNA-Seq analyses revealed a total of 768 DE ( $q < 0.10$ ) in LM. A total of 327 DE genes had higher expression in low tADG pigs, and 441 DE genes had lower expression in low tADG pigs (i.e., higher expression in high tADG pigs). No DE genes ( $q < 0.10$ ) were identified in adipose tissue samples.

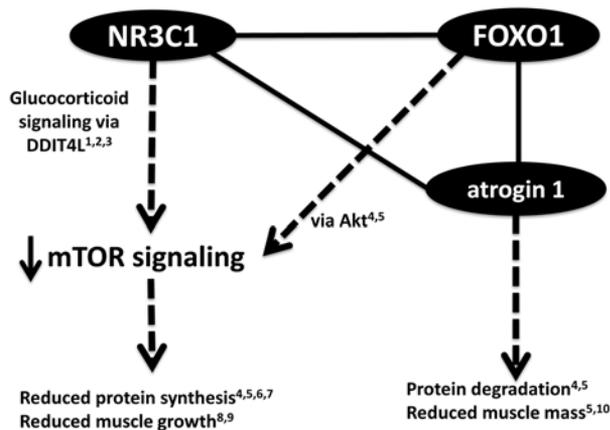
### RNA-Seq Data Validation by Quantitative PCR

To verify expression patterns measured by RNA-Seq in LM, we used quantitative PCR (qPCR) to assay mRNA levels for 32 genes, which were predicted to be DE between high and low tADG pigs ( $q < 0.10$ ). For all genes tested for validation by qPCR, differences in expression between the high and low tADG pigs were consistent in direction with the RNA-Seq results (Supplementary Table 3). Statistical significance ( $FDR < 0.10$ ) was confirmed by qPCR in 90% of the tested genes in LM.

### Gene Ontology Enrichment Analysis in LM

To identify biological pathways potentially underlying the effects of tADG on skeletal muscle metabolism and physiology, we performed a functional enrichment analysis of genes DE between high and low tADG pigs using DAVID (Supplementary Tables 4 and 5). Overrepresentation of GO Biological Process terms was determined for the list of DE genes, and 113 processes were identified, 79 by genes with lower expression in low tADG pigs and 32 by genes with higher expression in low tADG pigs ( $P < 0.05$ ).

Terms with significant similarity were further clustered into 8 significant annotation clusters (enrichment scores  $> 1.3$ ; Table 2) for DE genes with lower



**Figure 1.** Pathway enrichment analysis of differentially expressed (DE) genes in LM indicates that forkhead box O1 (FOXO1) and the glucocorticoid receptor (NR3C1) may play a role in differences in low and high transition ADG (tADG) pigs. These results are consistent with the known roles of FOXO1 and glucocorticoid signaling through mechanistic target of rapamycin (mTOR), a major regulator of protein synthesis. It is known that NR3C1 and FOXO1 interact and that FOXO1 influences glucocorticoid signaling via atrogin 1 to result in protein degradation and muscle atrophy. Solid lines represent interactions based on Pathway Studio (NR3C1 and FOXO1) and the literature (FOXO1 and atrogin 1). Dashed lines represent inferred relationships based on and biological outcomes predicted on the basis of previous studies. Both *FOXO1* and *atrogin 1* were DE ( $q < 0.10$ ) and expressed higher in low vs. high tADG pigs. The *NR3C1* gene was not DE, but its targets were DE and were identified as enriched by Pathway Studio. References are denoted by superscripts: 1, Corradetti et al. (2005); 2, Miyazaki and Esser (2009); 3, Schakman et al. (2008); 4, Stitt et al. (2004); 5, Kamei et al. (2004); 6, Conn and Qian (2011); 7, Wang and Proud (2006); 8, Schakman et al. (2013); 9, Ge and Chen (2012); 10, Wu et al. (2008).

expression in low tADG pigs. These clusters showed the following enriched functions: muscle contraction, glucose metabolic process, cytoskeleton organization, actin filament-based movement, angiogenesis, muscle development, glycogen metabolism, and response to hormone stimulus. Differentially expressed genes with higher expression in low tADG LM produced 1 cluster with overrepresentation of GO terms related to protein catabolic process (enrichment scores  $< 1.3$ ; Table 3).

Analysis of KEGG pathways using DAVID revealed overrepresentation of glycolysis/gluconeogenesis (FDR  $< 0.001$ ) in DE genes with lower expression in low tADG pigs and overrepresentation of the proteasome (FDR  $< 0.01$ ) in DE genes with higher expression in low tADG pigs (Supplementary Table 6).

### Pathway Studio Analysis of DE Genes

To understand and identify possible regulators of the pathways identified by DE gene analysis, DE gene lists were analyzed for their connections to common transcription factors, nuclear receptor regulators, and other genes by using Pathway Studio (Nikitin et al., 2003). Connections were identified on the basis of literature evidence of at least 1 of 4 interaction catego-

ries provided by Pathway Studio, which were promoter binding, direct regulation, regulation, and expression (Table 4 and Supplementary Table 7). Candidate hub genes identified by Pathway Studio were declared significant if the list of genes connected to them was significantly enriched above background ( $P < 0.05$ ).

The most connected gene hub for DE genes with higher expression in low tADG pigs was the nuclear receptor subfamily 3, group C, member 1 (*NR3C1*; 11 connected DE genes). The *NR3C1* gene encodes the glucocorticoid receptor, which is the exclusive mediator of physiological effects of glucocorticoids in skeletal muscle (Reyer et al., 2013). Prolactin (*PRL*), cyclic adenosine monophosphate (cAMP) responsive element binding protein 1 (*CREB1*), and janus kinase 2 (*JAK2*) were also identified as highly connected with DE genes with higher expression in low tADG LM.

The most connected gene hub for DE genes with lower expression in LM of low tADG pigs was growth hormone (*GHI*; 13 connected DE genes). Thyroid stimulating hormone (*TSH*), forkhead box O1 (*FOXO1*), androgen receptor (*AR*), forkhead box M1 (*FOXM1*), myeloblastosis transcription factor (*MYB*) transcription factor (*MYB*), and peroxisome proliferator-activated receptor delta (*PPARD*) were also identified as highly connected to those DE genes with lower expression in LM of low tADG pigs.

Figure 1 provides a summary of the connections between GO enrichment and Pathway Studio analyses in muscle.

All supplementary materials supporting this manuscript are available online at the JAS website at <https://www.animalsciencepublications.org/publications/jas>

## DISCUSSION

Changes in gene expression and metabolic pathways in LM were observed among pigs in the 10th vs. the 60th to 70th percentile of postweaning ADG. There was a greater than 2-fold difference in ADG during the 3-wk period following weaning. Although alterations in RNA expression do not always correspond to alterations in protein abundance or activity, systematic changes in the expression of groups of genes that have overrepresented GO terms, KEGG pathways, or Pathway Studio categories indicate potential functional changes in the respective pathways or biological processes.

We understand that differences in gene expression between high and low tADG pigs may be the result of upregulation or downregulation in 1 phenotype or both. For the sake of clarity, and to focus specifically on the objective of this study (i.e., to investigate the molecular basis of poor postweaning growth perfor-

**Table 2.** Functional annotation clusters and corresponding Gene Ontology (GO) biological process terms for differentially expressed (DE) genes with lower expression ( $q < 0.10$ ) in longissimus dorsi tissue of low transition ADG pigs<sup>1</sup>

Cluster ES	GO Biological Process Term	Number of DE Genes	GO Term <i>P</i> -value
Cluster 1			
6.29	GO:0006936~muscle contraction	18	<0.0001
	GO:0003012~muscle system process	18	<0.0001
	GO:0006941~striated muscle contraction	9	<0.0001
Cluster 2			
3.42	GO:0006006~glucose metabolic process	18	<0.0001
	GO:0019318~hexose metabolic process	18	<0.0001
	GO:0006090~pyruvate metabolic process	9	<0.0001
	GO:0006094~gluconeogenesis	7	<0.0001
	GO:0005996~monosaccharide metabolic process	18	<0.0001
	GO:0019319~hexose biosynthetic process	7	0.0001
	GO:0034637~cellular carbohydrate biosynthetic process	9	0.0004
	GO:0046364~monosaccharide biosynthetic process	7	0.0005
	GO:0044275~cellular carbohydrate catabolic process	9	0.0006
	GO:0006096~glycolysis	7	0.0006
	GO:0046165~alcohol biosynthetic process	7	0.001
	GO:0046164~alcohol catabolic process	8	0.002
	GO:0006007~glucose catabolic process	7	0.002
	GO:0016052~carbohydrate catabolic process	9	0.003
	GO:0019320~hexose catabolic process	7	0.004
GO:0046365~monosaccharide catabolic process	7	0.005	
GO:0016051~carbohydrate biosynthetic process	9	0.007	
Cluster 3			
2.53	GO:0007010~cytoskeleton organization	25	0.0003
	GO:0030029~actin filament-based process	16	0.002
	GO:0030036~actin cytoskeleton organization	12	0.04
Cluster 4			
2.40	GO:0030048~actin filament-based movement	6	0.0002
	GO:0033275~actin-myosin filament sliding	4	0.001
	GO:0070252~actin-mediated cell contraction	4	0.001
	GO:0030049~muscle filament sliding	4	0.001
	GO:0030705~cytoskeleton-dependent intracellular transport	6	0.01
Cluster 5			
2.31	GO:0048514~blood vessel morphogenesis	14	0.003
	GO:0001568~blood vessel development	15	0.004
	GO:0001944~vasculature development	15	0.005
	GO:0001525~angiogenesis	10	0.01
Cluster 6			
1.56	GO:0007517~muscle organ development	17	0.0001
	GO:0060537~muscle tissue development	12	0.0004
	GO:0014706~striated muscle tissue development	11	0.001
	GO:0060538~skeletal muscle organ development	6	0.04
	GO:0007519~skeletal muscle tissue development	6	0.04
	GO:0031032~actomyosin structure organization	4	0.04
Cluster 7			
1.35	GO:0005977~glycogen metabolic process	5	0.01
	GO:0044042~glucan metabolic process	5	0.01
	GO:0006073~cellular glucan metabolic process	5	0.01
	GO:0006112~energy reserve metabolic process	5	0.02
	GO:0044264~cellular polysaccharide metabolic process	5	0.05
Cluster 8			
1.31	GO:0032868~response to insulin stimulus	9	0.004
	GO:0043434~response to peptide hormone stimulus	11	0.005
	GO:0032869~cellular response to insulin stimulus	6	0.03

<sup>1</sup>An enrichment score (ES) of >1.3 is equivalent to a *P*-value of <0.05. The Fisher exact *P*-value represents the degree of enrichment of the GO term.

**Table 3.** Functional annotation cluster and corresponding Gene Ontology (GO) biological process terms for differentially expressed (DE) genes with higher expression ( $q < 0.10$ ) in longissimus dorsi tissue of low transition ADG pigs<sup>1</sup>

Cluster ES	GO Biological Process Term	Number of DE Genes	GO Term <i>P</i> -value
1.44	GO:0006511~ubiquitin-dependent protein catabolic process	12	0.01
	GO:0030163~protein catabolic process	22	0.01
	GO:0051603~proteolysis involved in cellular protein catabolic process	21	0.02
	GO:0044257~cellular protein catabolic process	21	0.02
	GO:0044265~cellular macromolecule catabolic process	24	0.02
	GO:0043632~modification-dependent macromolecule catabolic process	20	0.02
	GO:0019941~modification-dependent protein catabolic process	20	0.02
	GO:0009057~macromolecule catabolic process	25	0.02

<sup>1</sup>An enrichment score (ES) of  $>1.3$  is equivalent to a *P*-value of  $<0.05$ . The Fisher exact *P*-value represents the degree of enrichment of the GO term.

mance), the discussion focuses on changes in gene expression and pathways in the low tADG pigs.

It is important to note that a PRRSv outbreak that occurred in wk 2 postweaning likely influenced the results of this experiment. All pigs in both high and low tADG groups were PCR positive for the North American genotype of PRRS in lung tissue at 3 wk postweaning (Jones et al., 2014). The PRRS virus is highly associated with secondary infections (Done and Paton, 1995; Zimmerman et al., 1997); however, there were no differences between tADG categories for the incidence of *Haemophilus parasuis*, porcine circovirus type 2 (PCV2), rotavirus, *Streptococcus suis*, or *Salmonella* spp. B infection or for immune marker concentrations (i.e., IgA, IL-1 $\beta$ , IL-8, and total glutathione) in serum or ileal mucosa at 3 wk postweaning for the population from which pigs in this experiment were selected (Jones et al., 2014).

It is also important to acknowledge that feed intake is likely a factor contributing to differences in tADG. Since this study focuses on findings in a commercial study, it was not possible to record individual pig feed intake data. The study was conducted under field conditions because this is the environment in

which the poor transitioning phenotype is thought to occur most often. Animals were housed in pens of 26 to 27 animals. Previous work from our research team did identify that feed intake was lower for poor transitioning pigs (Jones et al., 2012). However, feed intake is likely not the only factor involved in tADG. The gene expression changes identified in this study provide a snapshot of the physiological differences in these animals due to varying levels of not only feed intake but also other factors such as animal health and maternal rearing effects.

#### *No Differentially Expressed Genes Were Identified in Adipose Tissue*

Previous research indicated that poor transitioning pigs had reduced whole-body lipid accretion rates (Jones et al., 2012). In the current experiment, the low tADG pigs had less whole body lipid content than the high tADG pigs. However, we were unable to detect any DE transcripts in subcutaneous back fat between the high and low tADG groups. Exploring transcript changes at different time points after weaning or different adipose tissue depots may identify DE genes.

**Table 4.** Pathway Studio subnetwork enrichment analysis to identify possible regulators of differentially expressed genes ( $q < 0.10$ ) in longissimus dorsi muscle of low and high transition ADG (tADG) pigs

Gene network hub	Gene network hub name	Number of connecting genes	Median fold change	Enrichment <i>P</i> -value
Higher expression in low tADG				
NR3C1	Glucocorticoid receptor	11	2.07	0.03
PRL	Prolactin	8	2.78	0.01
CREB1	cyclic adenosine monophosphate responsive element binding protein 1	7	2.38	0.03
JAK2	Janus kinase 2	5	3.74	0.02
Lower expression in low tADG				
GH1	Growth hormone	13	-1.54	0.03
TSH	Thyroid stimulating hormone	11	-1.39	0.03
FOXO1	Forkhead box O1	10	-1.55	0.04
AR	Androgen receptor	8	-1.52	0.01
FOXM1	Forkhead box M1	8	-1.52	0.01
MYB	myeloblastosis transcription factor	6	-1.77	0.001
PPARD	Peroxisome proliferator-activated receptor delta	5	-1.60	0.01

### **Expression Differences in Genes Involved in Energy Utilization in Muscle**

Poor weaning transition in pigs is often associated with low feed intake (Huang et al., 2011; Jones et al., 2012). Undernourished animals are capable of altering metabolism to prioritize glucose for the brain and other obligate users of glucose. One possible way that glucose utilization in muscle may be reduced is through decreased transcription of genes encoding glycolytic enzymes. We observed reductions in the mRNA levels for several key glycolytic genes in the LM of low tADG pigs, including glucose-6-phosphate isomerase (*GPI*), phosphoglycerate kinase (*PGKI*), phosphoglycerate mutase (*PGAM2*), and pyruvate kinase (*PKM*;  $q < 0.10$ ). We also observed lower expression of genes involved in glycogen metabolism in LM of low tADG pigs, including glycogen synthase (*GYS1*), glycogen phosphorylase (*PYGM*), phosphorylase kinase (*PHKA1*), and the glycogen debranching enzyme amylo- $\alpha$ -1, 6-glucosidase, 4- $\alpha$ -glucanotransferase (*AGL*). These results indicate that glucose utilization and turnover are likely reduced in skeletal muscle of the low tADG pigs.

The AMP-activated protein kinase (*AMPK*) system plays an important role in regulation of cellular energy balance and in whole-body energy metabolism. Elevation of *AMPK* is associated with energy need. Phosphorylation of acetyl CoA carboxylase by *AMPK* attenuates de novo fatty acid synthesis in response to low energy status. Thus, activation of *AMPK* in skeletal muscle leads to increased fatty acid oxidation and glucose uptake. The *AMPK* protein is heterotrimeric, consisting of a catalytic ( $\alpha$ ) and 2 regulatory ( $\beta$  and  $\gamma$ ) subunits. In our experiment, genes encoding *AMPK*  $\alpha$ 2 (*PRKAA2*) and *AMPK*  $\beta$ 2 (*PRKAB2*), the main catalytic and regulatory isoforms in skeletal muscle, respectively, had lower expression in LM of low tADG pigs.

Hormone-sensitive lipase (*HSL*), the major enzyme mediator of the lipolytic pathway, had higher mRNA expression in LM of low tADG pigs. Although *HSL* activity is controlled posttranslationally through phosphorylation (Yeaman, 1990), there is evidence that prolonged feed deprivation results in an increase in *HSL* mRNA in skeletal muscle tissue in the pig (Spurlock et al., 2001). Fatty acids liberated by *HSL* may be utilized as an energy source within the muscle via  $\beta$  oxidation.

### **GO Term Clusters Indicate Possible Changes in Protein Turnover in Muscle**

The low tADG pigs had lower expression of genes related to skeletal muscle structure and function, including actin,  $\alpha$  1 (*ACTA1*), myomesin 2 (*MYOM2*), tropomyosin 1,  $\alpha$  (*TPMI*), myosin light chain 1 (*MYL1*), and 6 myosin heavy chain isoforms, including IIA, IIB, and

IIX ( $q < 0.10$ ). Previous research indicated that poor transitioning pigs have lower rates of protein accretion than their contemporaries (Jones et al., 2012), and gene expression data from the current study indicate that the reduced rate of protein accretion may be driven by lower gene expression related to both synthesis and degradation of skeletal muscle protein.

The ubiquitin-proteasome system is a major contributor to protein degradation in skeletal muscle. Several genes involved in ubiquitin-dependent catabolism, including genes that encode ubiquitin-specific peptidases, ATPase and non-ATPase components of the 26S proteasome subunit, and the F-box only protein 32 (*FBXO32*), had higher expression in low tADG pigs ( $q < 0.10$ ). The *FBXO32* protein, also known as MAFbx or atrogin 1, is the substrate recognition component of a muscle-specific E3 ubiquitin ligase. Atrogin 1 binds to target proteins to induce ubiquitin binding and degradation through the proteasome. Atrogin 1 is a key marker of the atrophy process and has been shown to be induced in multiple models of skeletal muscle atrophy resulting from a variety of conditions, including muscle inactivity, multiple disease states, and starvation (Schiaffino et al., 2013).

The major pathway regulating muscle protein synthesis is the mechanistic target of rapamycin (*mTOR*) pathway. Genes that encode 2 negative regulators of *mTOR* signaling, the eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*) and DNA-damage-inducible transcript 4-like (*DDIT4L*), had higher expression in low tADG pigs ( $q < 0.10$ ). When anabolic signaling is low, the *EIF4E-BP1* protein represses translation initiation by binding to eIF4E, blocking eIF4E-eIF4G complex formation. When anabolic signaling via *mTOR* is high, *4E-BP1* is phosphorylated, and eIF4E is released, which can then bind to eIF4G, forming a complex that upregulates mRNA binding to the 40S ribosomal subunit, thereby increasing translation initiation (Davis et al., 2008). Transcriptional control of *4EBP* has not been extensively evaluated; however, because of its function as a negative regulator of translational initiation, higher expression may lead to reduced protein synthesis. The protein encoded by the *DDIT4L* gene, *REDD2*, suppresses anabolic signaling upstream of *mTOR* and has been shown to inhibit *mTOR* signaling because of a variety of stress-related responses, including DNA damage, hypoxia, and glucocorticoid treatment (Corradetti et al., 2005; Miyazaki and Esser, 2009).

These alterations in gene expression related to muscle protein turnover indicate a possible repartitioning of amino acids away from muscle in low tADG pigs. Protein synthesis is energetically costly and can be slowed as a survival mechanism during periods of low

feed intake. Amino acids resulting from the breakdown of skeletal muscle tissue can be mobilized and redistributed for maintenance of more critical tissues in a period of low feed intake. The amino acids can also be oxidized and utilized as an energy source for the liver and other key tissues in the body during times of energy shortage.

### ***FOXO1: Key Link Between Protein Synthesis and Degradation and Energy Metabolism***

The forkhead box O transcription factor *FOXO1* had higher expression in the low tADG pigs ( $q < 0.10$ ). Muscle protein synthesis (via Akt-mTOR signaling) is linked to muscle ubiquitin-dependent protein degradation and muscle atrophy via FOXO. In the presence of insulin or IGF-I, the phosphorylation via Akt pathway signaling sequesters FOXO transcription factors in the cytoplasm (Stitt et al., 2004). In the absence of insulin or IGF-I, Akt is inactive, and FOXO proteins are translocated to the nucleus and induce the transcription of target genes that regulate protein degradation (Kamei et al., 2004). F-box only protein 32 is a key transcriptional target of FOXO1. Overexpression of FOXO1 in transgenic mice showed markedly reduced muscle mass (Kamei et al., 2004). Forkhead box O knockdown blocks the upregulation of FBXO32 (atrogin 1), preventing muscle loss during atrophy (Sandri et al., 2004). Interestingly, FOXO1 was identified by our Pathway Studio analysis because of its negative association with several genes with higher expression in high tADG pigs. The FOXO genes also mediate glucocorticoid-induced muscle protein catabolism (Schakman et al., 2008). The glucocorticoid receptor, NR3C1, was identified as a putative regulator by Pathway Studio. These results indicate that there are transcription changes in the *FOXO1* regulator and glucocorticoid pathway that may play a role in muscle protein catabolism. Gene and pathway enrichment results in this study indicate that changes in muscle protein growth and breakdown maybe mediated through FOXO1, NR3C1, and mTOR signaling.

### ***Conclusions***

This study identified differences in transcript abundance in the LM but not in adipose tissue related to the reduced growth in low tADG pigs. Changes in transcript abundance in LM indicate a possible shift in low tADG pigs to decreased protein synthesis, increased protein degradation, and reduced glucose metabolism. These responses are consistent with gene expression differences in insulin and GH signaling and increased glucocorticoid signaling in the LM of low tADG pigs. We identified DE transcripts in the FOXO1 and the NR3C1 glucocorticoid receptor pathways that are con-

sistent with this concept. Many of the gene expression differences between poor transitioning pigs and their contemporaries may be related to the limited availability of energy and amino acids due to low feed intake. Interestingly, we did not detect any differences in gene expression in subcutaneous back fat tissue, which may suggest that changes in transcript levels in muscle are more relevant to understanding variation in tADG. However, further studies are needed to determine if genetics, management, and nutritional solutions can help to improve the growth rate of low tADG pigs.

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