

Article

A Systems Biology Approach Using Transcriptomic Data Reveals Genes and Pathways in Porcine Skeletal Muscle Affected by Dietary Lysine

Taiji Wang, Jean M. Feugang, Mark A. Crenshaw, Naresh Regmi, John R. Blanton Jr. and Shengfa F. Liao *

Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS 39762, USA; tw1257@msstate.edu (T.W.); jn181@msstate.edu (J.M.F.); mac4@msstate.edu (M.A.C.); nr388@msstate.edu (N.R.); jblanton@ads.msstate.edu (J.R.B.J.)

* Correspondence: s.liao@msstate.edu; Tel.: +1-662-325-7318

Abstract: Nine crossbred finishing barrows randomly assigned to 3 dietary treatments were used to investigate the effects of dietary lysine on muscle growth related metabolic and signaling pathways. Muscle samples were collected from the *longissimus dorsi* of individual pigs after feeding the lysine-deficient, lysine-adequate, or lysine-excess diet for 5 weeks, and the total RNA was extracted afterwards. Affymetrix Porcine Gene 1.0 ST Array was used to quantify the expression levels of 19,211 genes. A total of 674 transcripts were differentially expressed ($P \leq 0.05$); 60 out of 131 transcripts ($P \leq 0.01$) were annotated in the NetAffx database. Ingenuity pathway analysis showed that dietary lysine deficiency may lead to (1) increased muscle protein degradation via the ubiquitination pathway as indicated by the up-regulated DNAJA1, HSP90AB1 and UBE2B mRNA, (2) reduced muscle protein synthesis via the up-regulated RND3 and ZIC1 mRNA, (3) increased serine and glycine synthesis via the up-regulated PHGDH and PSPH mRNA, and (4) increased lipid accumulation via the up-regulated ME1, SCD, and CIDEA mRNA. Dietary lysine excess may lead to (1) decreased muscle protein degradation via the down-regulated DNAJA1, HSP90AA1, HSPH1, and UBE2D3 mRNA, and (2) reduced lipid biosynthesis via the down-regulated CFD and ME1 mRNA.

Keywords: lysine; skeletal muscle; transcriptome; gene expression; microarray; pig

1. Introduction

The growth and development of skeletal muscle of pigs essentially requires dietary supply of proteins, or amino acids (AAs), to be exact. Of the 20 AAs that serve as building blocks for protein biosynthesis, lysine makes up the biggest part of the body protein gain of growing-finishing pigs (7.1 g lysine per 100 g body protein) [1], but lysine is typically the first limiting AA in conventional grain based swine diets [1,2]. In the meantime, lysine is a truly essential AA for pig's life because it cannot be *de novo* synthesized from any other nutrients or nutrient metabolites within pig's body. Therefore, dietary inclusion of sufficient lysine is a must to optimize pig's growth performance.

It has been reported that dietary lysine supplementation can improve pig's growth performance, especially the retention of nitrogen [3,4]. Dietary deficiency of lysine can affect pig carcass characteristics by decreasing the lean meat percentage [5], increasing the subcutaneous fat depth [5,6], and increasing the intramuscular fat content of *longissimus dorsi* muscle [5-7]. However, the underlying molecular mechanisms by which dietary lysine regulates muscle protein accretion and fat deposition in pigs are still not clear.

Recent research advances revealed a key role AAs play as nutritional signals in regulation of cell signaling processes [8]. For example, leucine has been found to activate the mammalian target of rapamycin (mTOR) pathway to stimulate the initiation of mRNA translation in skeletal muscle of mammals [9]. Similarly, dietary arginine supplementation increased the activity of mTOR signaling

in skeletal muscle of neonatal pigs [10]. Glutamine may prevent protein hypercatabolism via inhibition of myostatin hyperexpression [11], and supplementation of glutamine to a high-fat diet improved insulin signaling in the muscle of rats [12]. In terms of lysine, however, which signaling pathways related to muscle protein accretion and carcass characteristics are regulated by its dietary supply is still unknown. Therefore, the objective of this study was to explore the effect of dietary lysine on the metabolic and cell signaling pathways associated with the growth performance, especially, the muscle growth, of finishing pigs.

As is known, muscle atrophy and sarcopenia are some common medical problems associated with human aging and cachexia-inducing diseases, such as cancer, AIDS, sepsis, and heart or renal disease [13-15]. Pigs seem to be the most appropriate, if not the best, animal model for human nutrition studies after non-human primates, simply because there are remarkable similarities between pigs and humans with respect to their nutritional requirements, as well as their digestive and metabolic processes [16-18]. The findings from this research project on pigs may also provide some novel insights into the molecular mechanisms of muscle growth vs. wasting in humans.

2. Results

The effects of dietary lysine at three different levels on the market-value associated carcass characteristics, along with some growth performance data, generated for this project were also the scientific interest of this research group and have been reported by Wang et al. [19]. This present report focuses on the effects of dietary lysine on skeletal muscle gene expression at the transcriptomic level.

2.1. Bioinformatics Analyses of Microarray Data

All the microarray *.cel files generated from the GeneChip Operating Software (GCOS; Affymetrix, Inc., Santa Clara, CA, USA) plus the GC Robust Multichip Averaging-corrected data processed with the Partek Genomics Suite (PGS) software (version 6.6; Partek Inc., St. Louis, MO, USA) have been deposited in the database of Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/geo>) following the standards of minimum information about a microarray experiment (MIAME) compliant [20]. The series accession number for the deposition assigned by the GEO is GSE77708.

The raw mRNA abundance values (i.e., the *.cel files) were imported into the PGS for ANOVA statistical analysis, which showed a total of 674 transcripts differentially expressed ($P \leq 0.05$) in the *longissimus dorsi* muscle of the pigs fed different diets. To refine this analysis, a subset of 131 transcripts with $P \leq 0.01$ were considered DEG (Supplemental Table S1). The overall dietary treatment effect on these 131 DEG is shown on Figure 1. Hierarchical cluster analysis of these DEG using PGS software showed that associated with the 3 dietary treatments are 3 clusters that have 3 distinct gene expression pattern (Figure 2). Of these 131 transcripts, 60 were annotated, 71 were unannotated in the NetAffx annotation database (Affymetrix), and the 60 annotated transcripts belong to 59 genes.

The orthogonal contrast analysis of the dietary treatments revealed a total of 40 genes differentially expressed in the skeletal muscle of the pigs fed Diet 1 vs. the pigs fed Diet 2, of which 28 genes were up-regulated and 11 were down-regulated (Table 1). Canonical pathway analysis of these 40 genes was conducted with the Core Analysis program of the Ingenuity Pathways Analysis (IPA, 8.0-2602) online software (<http://www.ingenuity.com>; Ingenuity Systems, Inc., Redwood City, CA, USA). The results showed that several cell signaling and metabolic pathways in skeletal muscle could have been affected ($P \leq 0.05$) by dietary deficiency of lysine (Figure 3), of which the top 5 ones are responsible for the serine and glycine biosynthesis pathways, protein ubiquitination pathway, and spermine and spermidine biosynthesis pathways.

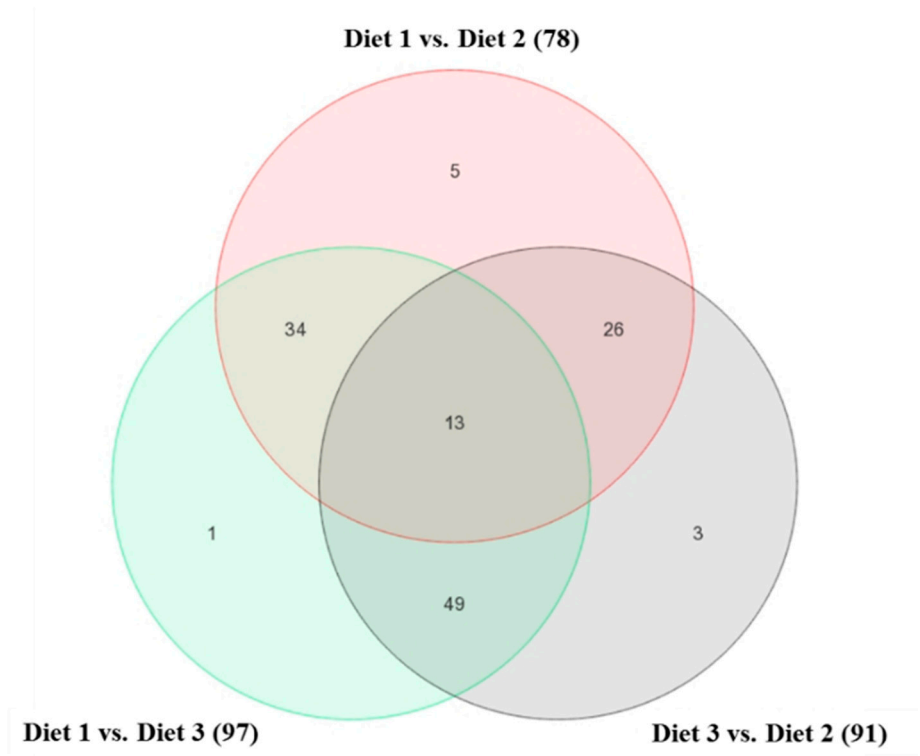


Figure 1. Venn diagram detailing the number of differentially expressed genes or gene transcripts (DEG; $P \leq 0.01$) in the skeletal muscle of finishing pigs fed 3 different diets. The number of DEG between each two dietary treatments is shown in parentheses.

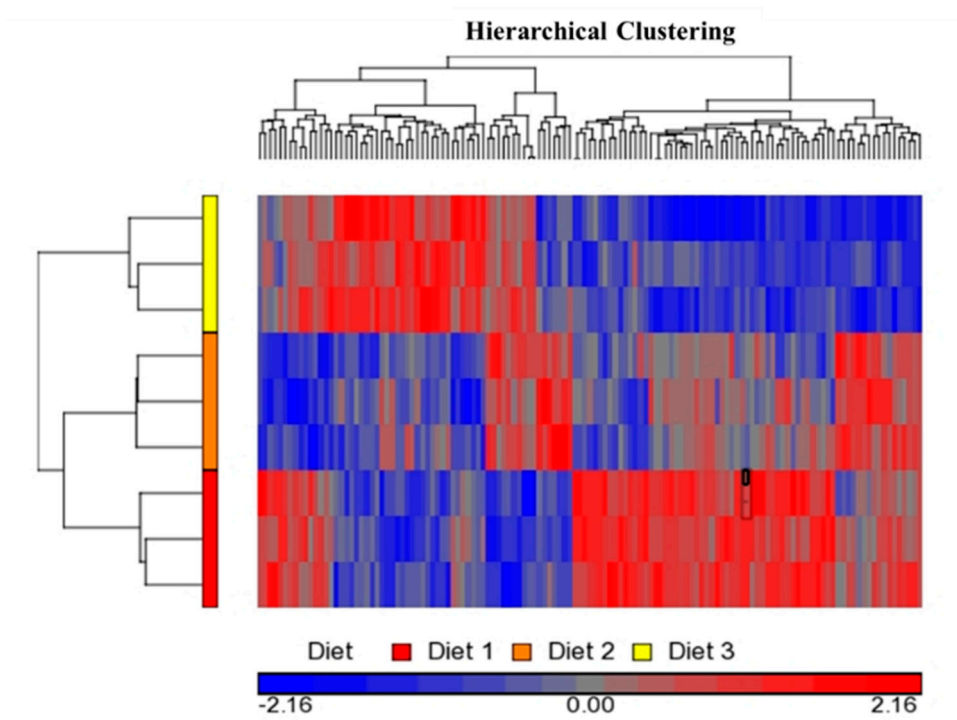


Figure 2. Hierarchical clusters of differentially expressed genes or gene transcripts (DEG; $P \leq 0.01$). The expression level for each gene transcript was standardized to mean of 0 and scale to SD of 1, which is the default setting of the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). As indicated by the legend color box, gray color in the middle represents the mean value, 0, red color

represents gene expression level above the mean, and blue color below the mean. The intensity of the color reflects the relative intensity of the fold change.

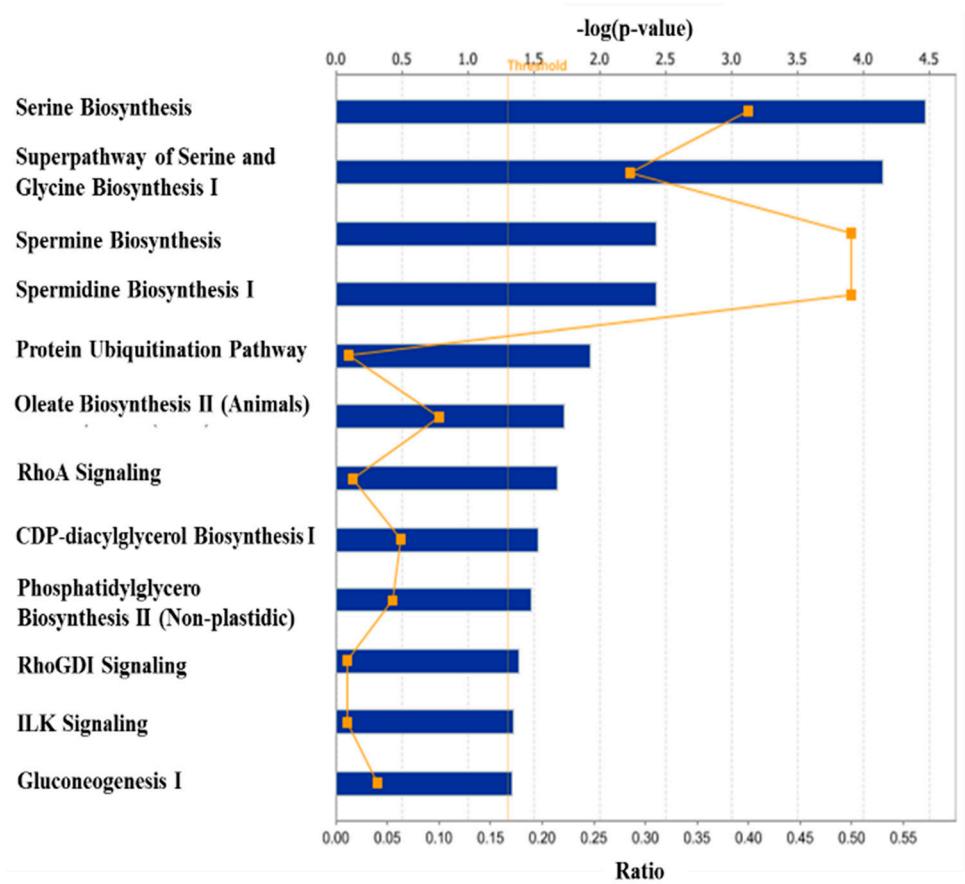


Figure 3. Canonical pathways significantly affected (a “-log of *P*-value” ≥ 1.3) by dietary lysine deficiency in skeletal muscle of finishing pigs. The x-axis displays the significant pathways, and the y-axis displays the -log of *P*-value. Each rectangular symbol connected with other rectangular symbols by a line represents a ratio that is calculated as the number of genes in a given pathway that meet the defined cutoff criteria divided by the total number of genes in the reference gene set that make up that pathway.

Table 1. Genes differentially expressed in the skeletal muscle of finishing pigs fed a lysine-deficient vs. a lysine-adequate diet.

Gene Symbol	Gene Description	GenBank ¹	P-value ²
<i>Down-regulated genes</i>			
CPEB2	Cytoplasmic polyadenylation element binding protein 2	NM_001185049	< 0.001
CST9L	Cystatin 9-like	XM_003134304	0.007
ERLEC1	Endoplasmic reticulum lectin 1	XM_003125147	0.011
ESYT1	Extended synaptotagmin-like protein 1	XM_003126262	0.009
HOXA11B	Homeobox A11	AF453292	0.001
LCLAT1	Lysocardiolipin acyltransferase 1	NM_001142845	0.032

MYL6	Myosin, light chain 6	NM_001163997	0.011
RASSF7	Ras association domain family member 7	XM_003122393	0.004
UBTD1	Ubiquitin domain containing 1	XM_003359315	0.003
ZMAT5	Zinc finger, matrin-type 5	XM_001929010	0.035
ZNF181	Zinc finger protein 181	NM_001244818	0.005
<i>Up-regulated genes</i>			
AMD1	Adenosylmethionine decarboxylase 1	XM_003121345	0.014
ATP6V1G2	ATPase, H ⁺ transporting, lysosomal 13 kDa, V1 subunit G2	NM_001145380	0.002
C7H6orf136	Chromosome 6 open reading frame 136	NM_001243459	0.003
CCDC25	Coiled-coil domain containing 25	NM_001243572	0.023
CD164	CD164 molecule	XM_001924626	0.022
CHIT1	Chitinase 1	XM_003130296	0.005
CHORDC1	Cysteine and histidine-rich domain (CHORD) containing 1	NM_001113446	0.010
CHSY3	Chondroitin sulfate synthase 3	XM_003123906	0.049
CIDEC	Cell death-inducing DFFA-like effector c	NM_001112689	0.024
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	NM_001244163	0.003
eIF2S2	Eukaryotic translation initiation factor 2 subunit 2	XM_005672861	0.002
FUT1	Fucosyltransferase 1	NM_214068	0.003
GABPB1	GA binding protein transcription factor, beta subunit 1	XM_005659610	0.002
GPR182	G protein-coupled receptor 182	XM_003126290	0.000
H1FOO	Oocyte-specific H1 histone	NM_001205063	0.016
HOXA4	Homeobox A4	XM_003134841	0.037
HSP90AB1	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	NM_001244433	0.007
LGALS13	Lectin, galactoside-binding, soluble, 13	NM_001142841	0.003
ME1	Malic enzyme 1, NADP ⁺ -dependent, cytosolic	XM_001924333	0.050
MIR3187	Microrna 3187	NR_036154	0.002
PHGDH	Phosphoglycerate dehydrogenase	NM_001123162	0.000
PSPH	Phosphoserine phosphatase	NM_001243221	0.009
RND3	Rho family GTPase 3	NM_214296	0.003
SCD	Stearoyl-coA desaturase (delta-9-desaturase)	NM_213781	0.023
SERP1	Stress-associated endoplasmic reticulum protein 1	NM_001243260	0.039
TXNL1	Thioredoxin-like 1	NM_001244276	0.030
UBE2B	Ubiquitin-conjugating enzyme E2B	NM_001257356	0.011
ZIC1	Zic family member 1	XM_003358599	0.011

¹ The accession number of the gene sequence resided in the GenBank database (<http://www.ncbi.nlm.nih.gov>).

² *P*-value was obtained from the orthogonal contrast analysis using the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). A change in gene mRNA abundance was considered significant when the *P*-value was less than or equal to 0.05.

The orthogonal contrast analysis of the dietary treatments also revealed a total of 35 genes differentially expressed in the skeletal muscle of the pigs fed Diet 3 vs. the pigs fed Diet 2, of which

13 genes were up-regulated and 22 were down-regulated (Table 2). Canonical pathway analysis of these 35 genes with the Core Analysis program of the IPA software showed that several cell signaling and metabolic pathways in the skeletal muscle of pigs might have been affected ($P \leq 0.05$) by dietary excess of lysine (Figure 4), of which the top 5 ones are responsible for protein ubiquitination pathway, spermine and spermidine biosynthesis pathways, CDP-diacylglycerol biosynthesis pathway, and phosphatidylglycerol biosynthesis.

Table 2. Genes differentially expressed in the skeletal muscle of finishing pigs fed a lysine-excess vs. a lysine-adequate diet.

Gene Symbol	Gene Description	GenBank ¹	P-value ²
<i>Down-regulated genes</i>			
ALKBH7	AlkB, alkylation repair homolog 7	XM_003123112	0.001
AMD1	Adenosylmethionine decarboxylase 1	XM_003121345	0.013
CFAP20	Cilia and flagella associated protein 20	NM_001244786	0.001
CFD	Complement factor D (adipsin)	XM_003122985	0.009
CHORDC1	Cysteine and histidine-rich domain (CHORD) containing 1	NM_001113446	0.006
CLCA2	Chloride channel accessory 2	XM_003125930	0.003
CNTFR	Ciliary neurotrophic factor receptor	XM_003130672	0.027
DNAJA1	Dnaj (Hsp40) homolog, subfamily A, member 1	NM_001244163	0.004
ERLEC1	Endoplasmic reticulum lectin 1	XM_003125147	0.004
HOXA4	Homeobox A4	XM_003134841	0.014
HSP90AA1	Heat shock protein 90kda alpha (cytosolic), class A member 1	NM_213973	0.034
HSPH1	Heat shock 105kda/110kda protein 1	NM_001097504	0.014
LCLAT1	Lysocardiolipin acyltransferase 1	NM_001142845	0.001
ME1	Malic enzyme 1, NADP+-dependent, cytosolic	XM_001924333	0.027
MFAP3	Microfibrillar-associated protein 3	XM_003134126	0.001
MORF4L2	Mortality factor 4 like 2	XM_003135267	0.033
SERP1	Stress-associated endoplasmic reticulum protein 1	NM_001243260	0.016
SLC9A2	Solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2	NM_001100189	0.010
TMCO6	Transmembrane and coiled-coil domains 6	XM_003124040	0.003
UBE2D2	Ubiquitin-conjugating enzyme E2D 2	NM_001078673	0.028
UBTD1	Ubiquitin domain containing 1	XM_003359315	0.009
ZMAT5	Zinc finger, matrin-type 5	XM_001929010	0.001
<i>Up-regulated genes</i>			
CHIT1	Chitinase 1 (chitotriosidase)	XM_003130296	0.009
CHSY3	Chondroitin sulfate synthase 3	XM_003123906	0.001
CNTROB	Centrobin, centrosomal BRCA2 interacting protein	XM_003358269	0.007
CXCR6	Chemokine (C-X-C motif) receptor 6	NM_001001623	0.000

DBX1	Developing brain homeobox 1	XM_003122916	0.002
DND1	DND microrna-mediated repression inhibitor 1	XM_003124043	0.009
GPR182	G protein-coupled receptor 182	XM_003126290	0.008
H1FOO	Oocyte-specific H1 histone	NM_001205063	0.003
LGALS13	Lectin, galactoside-binding, soluble, 13	NM_001142841	0.005
MYO5B	Myosin VB	XM_003121434	0.001
PDCL	Phosducin-like	XM_001927696	0.003
XKR4	XK, Kell blood group complex subunit-related family, member 4	XM_003355057	0.008
ZNF181	Zinc finger protein 181	NM_001244818	0.037

¹ The accession number of the gene sequence resided in the GenBank database (<http://www.ncbi.nlm.nih.gov>).

² *P*-value was obtained from the orthogonal contrast analysis using the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). A change in gene mRNA abundance was considered significant when the *P*-value was less than or equal to 0.05.

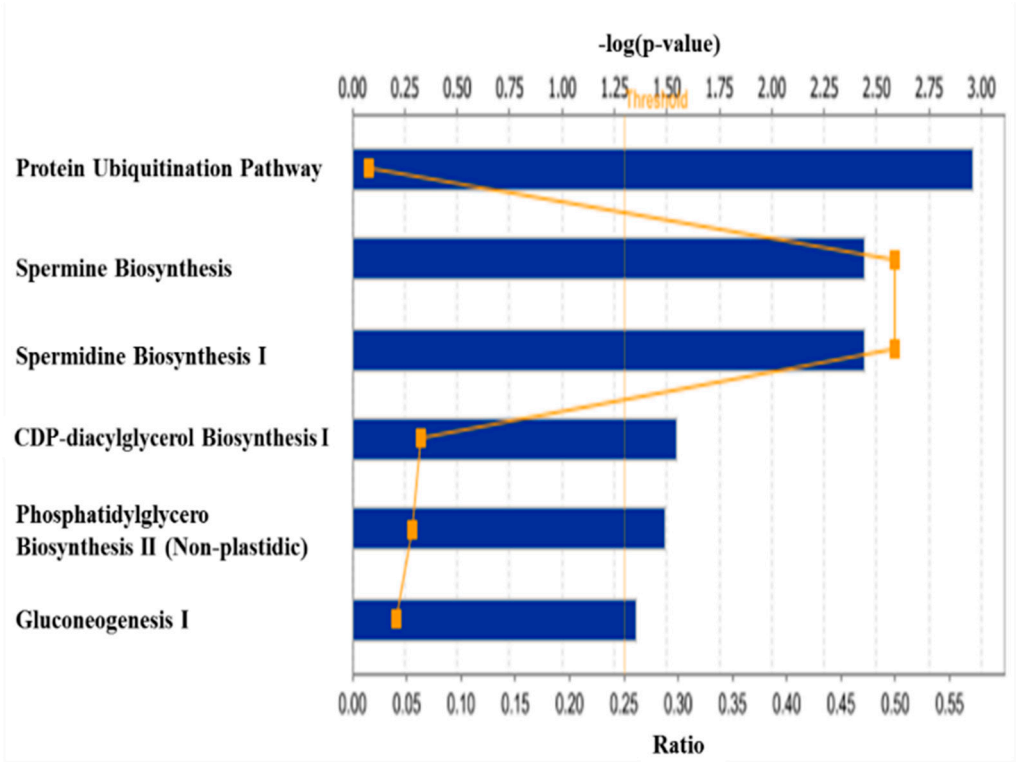


Figure 4. Canonical pathways significantly affected (a “-log of *P*-value” ≥ 1.3) by dietary lysine deficiency in skeletal muscle of finishing pigs. The x-axis displays the significant pathways, and the y-axis displays the -log of *P*-value. Each rectangular symbol connected with other rectangular symbols by a line represents a ratio that is calculated as the number of genes in a given pathway that meet the defined cutoff criteria divided by the total number of genes in the reference gene set that make up that pathway.

2.2. Real-time RT-PCR Analysis of Selected Genes

Nine genes involved in serine and glycine biosynthesis, lipid biosynthesis, spermine and spermidine biosynthesis, and protein ubiquitination pathway, identified from the bioinformatics analyses of the microarray data, were selected for validation using semi-quantitative real-time RT-PCR analysis, and these genes were PHGDH (phosphoglycerate dehydrogenase), PSPH (phosphoserine phosphatase), SCD (stearoyl-CoA desaturase), CIDEA (Cell death-inducing DFFA-like effector c), AMD1 (adenosylmethionine decarboxylase 1), ZIC1 (Zic family member 1), ERLEC1 (endoplasmic reticulum lectin 1), DNAJA1 (DnaJ (Hsp40) homolog subfamily A member 1), and ZNF181 (Zinc finger protein 181).

As shown in Figure 5, the levels of PHGDH, PSPH, SCD, CIDEA and ZIC1 mRNA expressed in the muscle of the pigs fed Diet 1 were significantly higher ($P \leq 0.05$) than those in the pigs fed Diet 2, and there were no differences in the mRNA levels of these five genes between the pigs fed Diet 3 vs. Diet 2 (Figure 5A, 5B, 5C, 5D and 5F). The level of the AMD1 mRNA expressed in the muscle of the pigs fed Diet 1 was higher ($P \leq 0.05$) than that in the pigs fed Diet 2, while its level in the pigs fed Diet 3 was lower ($P \leq 0.05$) than that in the pigs fed Diet 2 (Figure 5E). All these results are consistent with the results obtained from the microarray analysis concerning these genes (Tables 1 and 2).

The level of ERLEC1 mRNA in the muscle of pigs fed Diet 1 was significantly lower ($P \leq 0.05$) than that of pigs fed Diet 2 (Figure 5G), while the expression level of DNAJA1 in the muscle of pigs fed Diet 1 was significantly higher ($P \leq 0.05$) than that of pigs fed Diet 2 (Figure 5H). The level of ZNF181 mRNA in the muscle of pigs fed Diet 3 was significantly higher ($P \leq 0.05$) than that of pigs fed Diet 2 (Figure 5I). These results were also consistent with the results obtained from the microarray analysis concerning these two genes (Table 1). When compared to Diet 2, the expression levels of ERLEC1 and DNAJA1 mRNA in the pigs fed Diet 3 and the expression level of ZNF181 mRNA in the pigs fed Diet 1 were all numerically decreased (Figure 5G, 5H and 5I), which is also in agreement with the results obtained from the microarray analysis concerning these two genes (Table 2).

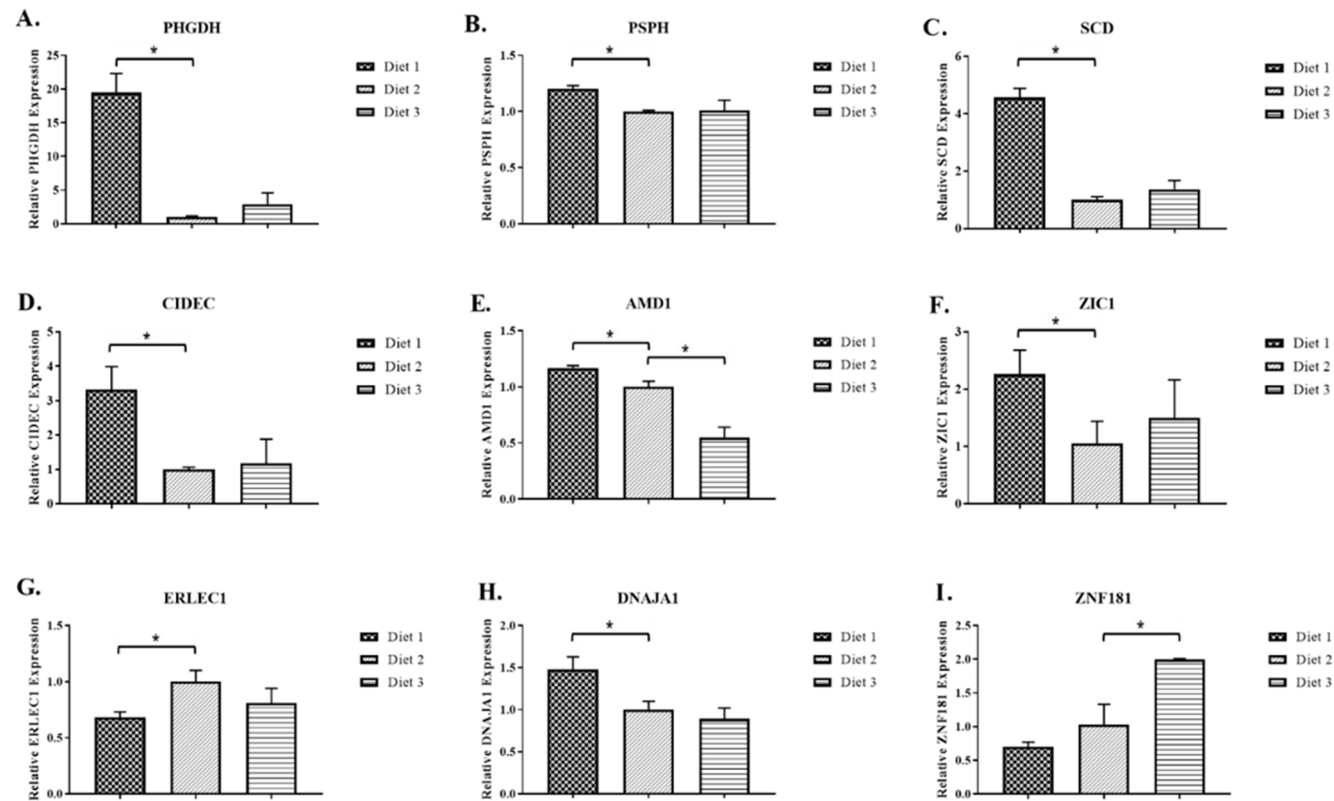


Figure 5. Semi-quantitative real-time RT-PCR analyses of PHGDH (A), PSPH (B), SCD (C), CIDEc (D), AMD1 (E), ZIC1 (F), ERLEC1 (G), DNAJA1 (H), and ZNF181 (I) mRNA expression in the skeletal muscle of pigs fed a lysine-deficient diet (D1), a lysine-adequate diet (D2), and a lysine-excess diet (D3). The y-axis shows the relative mRNA expression levels relative to the D2 pig group. The error bars denote SD, and the * signs denote differential expressions ($P \leq 0.05$). PHGDH = Phosphoglycerate dehydrogenase; PSPH = Phosphoserine phosphatase; SCD = Stearoyl-CoA desaturase; CIDEc = Cell death-inducing DFFA-like effector c; AMD1 = Adenosylmethionine decarboxylase 1; ZIC1 = Zic family member 1; ERLEC1 = Endoplasmic reticulum lectin 1; DNAJA1 = DnaJ (Hsp40) homolog, subfamily A, member 1; ZNF181 = Zinc finger protein 181.

3. Discussion

To the best of our knowledge, this study was the first to explore the transcriptomic profile of skeletal muscle of finishing pigs in response to dietary lysine levels. Approximately 130 genes or gene transcripts involved in different signaling and metabolic pathways were found to be affected by dietary lysine. Of these pathways, the significant ones are those associated with protein and lipid metabolism and muscle growth.

3.1. Regulation of Protein Turnover

3.1.1. Protein Degradation

It has been shown that dietary lysine may regulate protein degradation in skeletal muscle of pigs [3]. Ubiquitin-proteasome signaling pathway plays important roles in the breakdown of myofibrillar protein in skeletal muscle [21], which involves two successive steps: conjugation of multiple ubiquitin moieties to the target protein and degradation of the polyubiquitinated protein by the 26S proteasome complex. In this study, it was shown that lysine might affect the protein ubiquitination pathway through regulating at least 6 genes, DNAJA1, HSP90AA1, HSP90AB1, HSPH1, UBE2B and UBE2D2. More specifically, when compared to the lysine-adequate pigs (i.e., the Diet 2 group), dietary lysine deficiency increased the mRNA levels of DNAJA1, HSP90AB1, and UBE2B (Table 1), while dietary lysine excess decreased the mRNA levels of DNAJA1, HSP90AA1, HSPH1, and UBE2D2 (Table 2). That the expression levels of the genes related to protein ubiquitination pathway were down-regulated with the increased level of dietary lysine implies that a high level of dietary lysine may suppress protein degradation and that dietary lysine deficiency may stimulate protein degradation through the ubiquitin-proteasome degradation pathway. This implication is supported by an *in vivo* study [22], which showed that dietary lysine supplementation inhibited the ubiquitin-proteasome pathway in the skeletal muscle of rats.

Cystatin 9-like (CST9L), a cysteine protease inhibitor, prevents or reduces the activity of a cysteine protease (a.k.a. cysteine-type endopeptidase), which is an enzyme that can degrade proteins in animal body. When present at a high level or when abnormally activated, cysteine proteases are thought to be involved in numerous pathophysiological processes, such as muscular dystrophy [23]. In this study, the expression or abundance of CST9L mRNA was down-regulated by the lysine-deficient diet (Table 1), which means that the activity of cysteine-type endopeptidase might have been enhanced, leading to an increased protein degradation in skeletal muscle.

The endoplasmic reticulum (ER) lectin 1 (ERLEC1) gene encodes XTP3-B lectin, an ER protein that can function as a negative regulator of ER-associated protein degradation (via its interaction with the membrane-associated ubiquitin ligase complex), and can protect newly synthesized immature polypeptides from premature degradation [24]. The level of ERLEC1 mRNA was down-regulated in skeletal muscle of pigs fed Diet 1 or 3. The connection between the expression level of ERLEC1 mRNA and protein degradation in skeletal muscle associated with dietary lysine level is not clear and needs further investigation.

Based on our previous report [19], lysine deficiency was shown to significantly decrease the average daily gain (ADG) in finishing pigs, while there is no significant difference in the ADG between the pigs fed the lysine-excess and lysine-adequate diets. Data from this study suggest that the increased muscle protein degradation might be the reason for the decreased ADG when pigs were fed a lysine-deficient diet. However, whether the decreased expression of DNAJA1, HSP90AA1, HSPH1, and UBE2D2 caused by the excess of dietary lysine (Table 2) can lead to significantly decreased muscle protein degradation warrants further investigation.

It was reported that DNAJA1 gene encodes Hsp40 (heat shock protein 40), which is a negative marker of meat tenderness [25]. Interestingly, in this study we found that the level of DNAJA1 mRNA was decreased along with the increasing levels of dietary lysine (Tables 1 and 2). Therefore, dietary lysine deficiency may negatively affect pork quality, while dietary lysine excess may improve pork tenderness via the regulation of DNAJA1 gene expression in skeletal muscle.

3.1.2. Protein Synthesis

Several genes associated with protein synthesis, such as RND3 (Rho family gtpase 3) and ZIC1 (Zic family member 1), were regulated by dietary lysine in this study. RND3 is a member of Rnd family, a sub-group of the Rho family of small GTP-binding proteins. The Rnd family is comprised of three proteins including Rnd1/Rho6, Rnd2/Rho7, and Rnd3/Rho8/RhoE [26]. Upregulation of RND3 expression inhibited cell proliferation, which is associated with PTEN/PI3K/Akt signaling pathway [27]. ZIC1 encodes a member of the ZIC family of C2H2-type zinc finger proteins, and is an activator of Wnt signaling [28]. Overexpression of ZIC1 resulted in inactivation of PI3K and MAPK signaling pathways, which can further regulate multiple downstream targets [29]. The PI3K/Akt signaling pathway has a number of downstream effectors including mTOR, which can affect the transcription of p70 or 4EBP1 and regulate protein synthesis [30].

The results of this study showed that dietary lysine deficiency up-regulated the expression levels of RND3 and ZIC1 mRNA, which might lead to inhibition of PI3K/Akt signaling pathway, causing inactivation of downstream effectors of mTOR pathway and resulting in less muscle protein synthesis. Sato et al. [31] reported that lysine could in part affect the Akt pathway in C2C12 murine myotubes, and that the protein synthesis may be increased by lysine through mTOR pathway. The lower ADG, lower lean cut, and reduced ham weight of the pigs fed the lysine-deficient diet reported by Wang et al. [19] may be in part due to the reduced muscle protein synthesis via the up-regulation of RND3 and ZIC1 gene expression.

The serine and glycine biosynthesis pathway consists of three sequential enzymatic reactions (Figure 6), which begins with the oxidation of glycolytic intermediate phosphoglycerate (3PG) to 3-phosphohydroxypyruvate (pPYR) with the concomitant reduction of NAD⁺ co-factor to NADH by an enzyme called phosphoglycerate dehydrogenase (PHGDH). Phosphoserine amino transferase (PSAT1) then uses the α -amino group of glutamate to transaminate pPYR yielding phosphoserine (pSER) and α -ketoglutarate (α KG). Finally, phosphoserine phosphatase (PSPH) dephosphorylates pSER to give serine, which will interconvert with glycine in animal body [32]. This study revealed that dietary lysine deficiency increased the expression of PHGDH and PSPH mRNA, responsible for coding 2 rate-limiting enzymes in the serine and glycine biosynthesis pathway (Figure 6), and these increases indicated a positive serine and/or glycine biosynthesis in the skeletal muscle of the finishing pigs. One of our previous studies showed that the plasma concentrations of serine (numerically) and glycine ($P \leq 0.05$) were both decreased in the pigs fed a lysine-deficient diet [33], and these decreases suggested that there might not be enough serine and glycine supply in the plasma, causing active serine and glycine biosyntheses in skeletal muscle. Since serine and glycine are building blocks for protein synthesis and substrates for creatine, purine, and pyrimidine generation [34], the change of PHGDH and PSPH mRNA levels implies that dietary lysine could affect muscle energy metabolism, and the biosyntheses of nuclear acids and proteins.

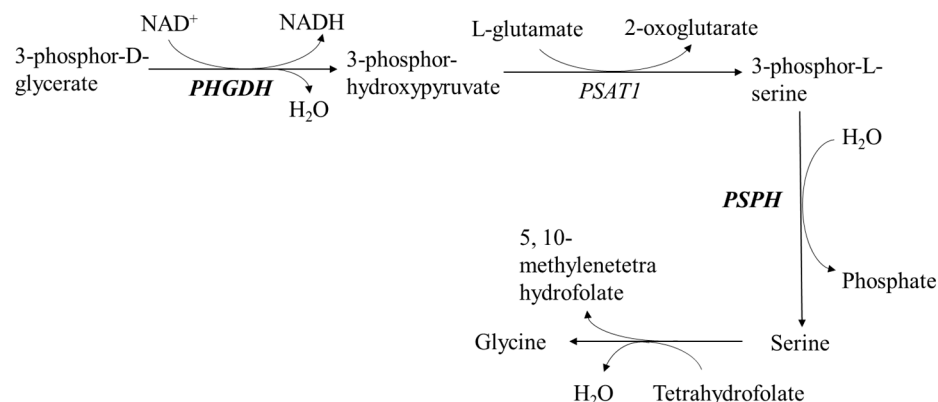


Figure 6. Roles of phosphoglycerate dehydrogenase (PHGDH) and phosphoserine phosphatase (PSPH) in the serine and glycine biosynthesis pathway (Adapted from Ingenuity Pathways Analysis (IPA, 8.0-2602) online software (<http://www.ingenuity.com>; Ingenuity Systems, Inc., Redwood City, CA, USA).

The biosynthesis pathways for two polyamines, spermine and spermidine, may be affected by dietary lysine through regulating the expression of AMD1, which encodes S-adenosylmethionine decarboxylase 1 (AdoMetDC), a key enzyme in the rate-limiting step of polyamine biosynthesis [35]. Although the potential mechanism by which polyamines regulate skeletal muscle hypertrophy and atrophy are still unclear, there is a strong association between polyamine levels and muscle mass [35,36]. The AMD1 mRNA level affected by dietary lysine may lead to a change in the polyamine level causing differential muscle growth. Regmi et al. [33] reported that the plasma concentration of arginine decreased along with the increased dietary lysine concentration. Since arginine is the main substrate for polyamine synthesis [37], the regulation of dietary lysine on AMD1 expression and then polyamine level may be due to the antagonism effect of lysine on plasma arginine level.

3.2. Regulation of Lipid Metabolism

Several DEG in Tables 1 and 2, including ME1 (malic enzyme 1), SCD (stearoyl-CoA desaturase), and CIDEC (cell death-inducing DFFA-like effector c), are associated with lipid biosynthesis. ME1 catalyzes the reversible oxidative decarboxylation of L-malate to pyruvate (Figure 7), which links glycolytic pathway and citric acid cycle, involving the reduction of NADP⁺ to NAD(P)H [38]. At the same time, a malic enzyme can form part of the tricarboxylate shuttle, which releases acetyl-CoA from the mitochondria into the cytosol [38]. Both NADPH and acetyl-CoA are substrates of fatty acid biosynthesis, and malic enzyme activity has a strong influence on intramuscular fat content [39]. In addition, a significant association between ME1 genotype and back-fat thickness of pigs has been reported and it was found that different malic enzyme activities are highly related to the fatness traits of different pig breeds [38]. Therefore, the increased level of ME1 mRNA in the skeletal muscle of the pigs fed the lysine-deficient diet might be associated with the enhanced lipid biosynthesis. Furthermore, the level of ME1 mRNA was decreased in pigs fed the lysine-excess diet. It seems that increasing dietary lysine level from deficiency to excess, the level of ME1 mRNA was decreased accordingly, which implies that ME1 enzyme may be a key regulator of lipid biosynthesis in response to dietary lysine. Specifically, dietary supplementation of lysine may inhibit lipid biosynthesis through the reduction of ME1 gene expression.

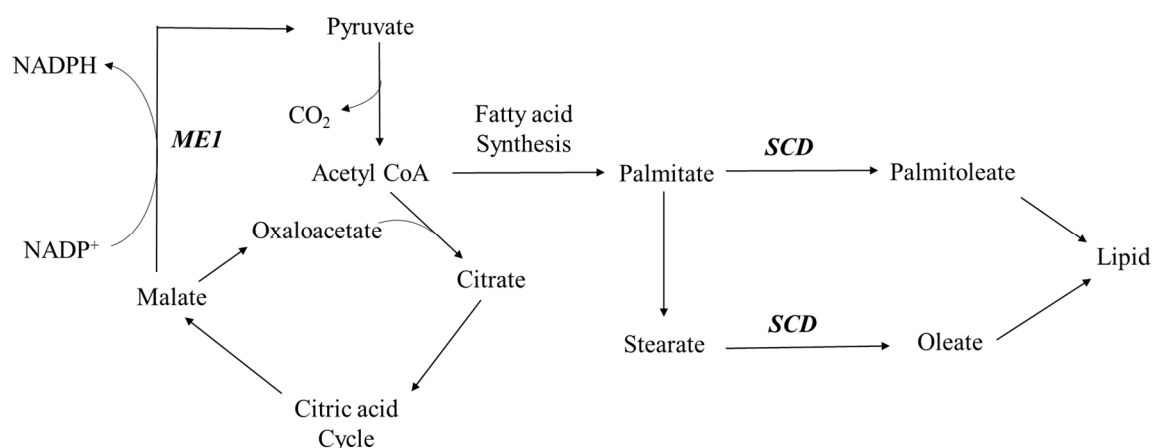


Figure 7. Roles of malic enzyme 1 (ME1) and stearoyl-CoA desaturase (SCD) in lipid biosynthesis pathway.

As shown in Figure 7, the end product of fatty acid synthesis from acetyl-CoA is palmitate, which can be elongated to stearate. SCD is the rate-limiting lipogenic enzyme in the biosynthesis of monounsaturated fatty acids [40]. It has been proven that the expression of SCD positively correlates with intramuscular fat deposition in pigs [41,42]. Also associated with body lipid accumulation is the CIDEC gene, and elevated CIDEC expression resulted in an inhibition of fatty acid oxidation and an increase of *de novo* lipogenesis in muscle cells [43]. Both SCD and CIDEC mRNA were up-regulated

in the lysine-deficient pigs, which is, at least partially, supported by da Costa et al. [42], who reported that when the dietary crude protein and lysine levels were low, the SCD mRNA expression was increased in the skeletal muscle of growing pigs.

The level of CFD (complement factor D, a.k.a. adipsin) mRNA in the skeletal muscle of finishing pigs was reduced after the lysine-excess diet was fed for 5 weeks (Table 2). As is known, CFD interacts with complement C3 and factor B to form acylation-stimulating protein, which is involved in stimulating glucose transport, enhancing fatty acid re-esterification, and inhibiting lipolysis [44]. Lan et al. [45] reported that a higher CFD level was associated with an increased fat content in skeletal muscle of the obese mice. The decreased CFD mRNA level in the lysine-excess group of this study may be associated with the reduced fatty acid synthesis and enhanced lipolysis, leading to a decreased fat deposition in the muscle when compared to the lysine-adequate pig group.

It has been well accepted that dietary lysine deficiency can lead to intramuscular fat accumulation in *longissimus dorsi* [5-7]. Our study implies that dietary lysine deficiency may promote lipid accumulation in skeletal muscle via increasing ME1, SCD, and CIDEA mRNA expression. It is interesting to mention that one of our previous studies [46] found that plasma concentration of cholesterol was significantly increased in pigs fed a lysine-deficient diet. As is known, SCD can catalyze the conversion of palmitate and stearate to their corresponding unsaturated fatty acids, which can be further used for the synthesis of cholesterol and phospholipid [40]. The active lipid accumulation in muscle and plasma might be due to lysine deficiency which restricts protein synthesis with surplus energy being converted to lipids. As aforementioned, dietary lysine deficiency stimulated serine synthesis, while serine serves as a substrate for synthesis of two membrane lipid molecules, phosphatidylserine and sphingolipids, which can be efficiently incorporated into cell membrane lipid bilayer [47]. Therefore, the increased serine synthesis in the lysine-deficient group may contribute to lipid biosynthesis as well.

Dietary lysine deficiency decreased the expression of the ESYT1 (extended synaptotagmin-like protein 1) mRNA, which encodes E-Syt1 (extended synaptotagmins). E-Syt1 are endoplasmic reticulum (ER) proteins that participate in tethering function between the ER and the plasma membrane (PM) and have roles in lipid transport between the two membranes [48]. The connection between ER and PM is dynamically regulated by Ca^{2+} signaling, of which elevation of cytosolic Ca^{2+} triggered translocation of E-Syt1 to ER-PM junctions to enhance ER-to-PM connection [49]. Dietary lysine supplementation has been shown to regulate Ca^{2+} metabolism [50], specifically enhancing intestinal Ca^{2+} absorption and improve the renal conservation of the absorbed Ca^{2+} . Therefore, the decreased level of ESYT1 mRNA in the lysine-deficient group of this study may be attributed to the effect of lysine on Ca^{2+} absorption, while at the same time lysine may affect lipid transport through E-Syt1.

Lysine may affect lipid oxidation through regulation of ALKBH7 (alkB, alkylation repair homolog 7), LGALS13 (lectin, galactoside-binding, soluble, 13), and LCLAT1 (lysocardiolipin acyltransferase 1) expression. ALKBH7 encodes a mitochondrial resident protein, which is located in the mitochondrial matrix and involved in fatty acid metabolism. ALKBH7 deletion has been shown to dramatically increase body fat and body weight, which may be due to the fact that ALKBH7 directly or indirectly facilitates the utilization of short-chain fatty acids [51]. LGALS13 encodes protein that has lysophospholipase activity, which was shown to be involved in phospholipid metabolism [52]. Deficiency of LCLAT1, which encodes Acyl-CoA : lysocardiolipin acyltransferase 1, has been shown to increase lipid oxidation and decrease the content of lipids in mouse [53]. Therefore, lysine may affect lipid content by regulating the expression of ALKBH7, LGALS13, and LCLAT1.

3.3. Other Biological Processes

The mRNA levels of several genes related to some other biological processes were also altered by the level of dietary lysine (Tables 1 and 2). Dietary lysine deficiency may affect the metabolism of macro molecules by increasing the expression of FUT1 (fucosyltransferase 1) and CHIT1 (chitinase 1), both of which are involved in the metabolic process of carbohydrates [54]. Dietary lysine deficiency increased the mRNA levels of TXNL1 (thioredoxin-like 1) and CD164 (an 80- to 100-kDa type 1

transmembrane sialomucin), encoding proteins involved in cell growth [55], of which TXNL1 may be regulated by Akt-mTOR pathway [56]. Lysine deficiency also decreased the expression of RASSF7 (Ras association domain family member 7), which is a member of the N-terminal Ras association domain family. RASSF7 negatively regulates pro-apoptotic JNK signaling [57], which is a member of an evolutionarily conserved sub-family of mitogen-activated protein (MAP) kinases and important as both positive and negative modulators of apoptosis [58]. Knocking down of RASSF7 has been shown to inhibit cell growth [59].

Dietary lysine-excess down-regulated the expression of SLC9A2 (solute carrier family 9, subfamily A, member 2), which is involved in the transport of Na⁺ and contributes to the regulation of intracellular pH [60]. Dietary lysine-excess increased the expression of CNTROB (centrobin, centrosomal BRCA2 interacting protein) and PDCL (phosducin-like), of which CNTROB is involved in centriole duplication [61], and PDCL has been shown to regulate G-protein signaling by binding to the beta-gamma subunits of G proteins [62].

In addition, several other genes related to gene transcription and protein translation in the skeletal muscle were also affected by dietary lysine. The genes encoding the class of transcription factors, called homeobox genes, are found in clusters named A, B, C, and D. HOXA11 and HOXA4 are part of the A cluster and encode DNA-binding transcription factors which may regulate gene expression. HOXA11 expression increases transcription of DNA [63] and was regulated by PTEN [64]. The function of ZNF181 (zinc finger protein 181) may be related to DNA binding and gene regulation [65]. ZMAT5 (zinc finger, matrin-type 5) is involved in mRNA splicing. MORF4L2 (MRGX) is a novel transcription factor and can repress or activate the B-myb promoter [66]. GABPB1 (GA binding protein transcription factor, beta subunit 1) encodes the GA-binding protein transcription factor, beta subunit, which forms a tetrameric complex with the alpha subunit, and influence the DNA binding stability of hGABP alpha and regulate hGABP-mediated transcription [67].

Translational control plays an essential role in regulating the expression of gene to mRNA and to protein. Members of CPEB (cytoplasm polyadenylation element binding protein) family have been shown to bind to the 3' UTR of target mRNAs and regulate their translation, of which CPEB2 interacts with the elongation factor, eEF2, to reduce the eEF2/ribosome-triggered GTP hydrolysis *in vitro* and slow down the peptide elongation of CPEB2-bound RNA *in vivo* [68]. Eukaryotic initiation factor 2 subunit 2 (eIF2S2), an initiation factor involved in mTOR pathway, is required in the initiation of protein translation and mediates the binding of tRNA^{met} to ribosome in a GTP-dependent manner. However, how the change of these mRNA abundance associated with dietary lysine would affect (inhibit or stimulate) transcription and translation warrants further investigation.

4. Materials and Methods

4.1. Animal Trial and Sample Collection

All the experimental protocols involving caring, handling, and treatment of pigs were approved by Mississippi State University Institutional Animal Care and Use Committee. A total of 9 crossbred (Large White × Landrace) late-stage finishing barrows (body weight 94.4 ± 6.7 kg) were housed in an environmentally controlled swine barn at the Leveck Animal Research Center, Mississippi State University. The pigs were randomly assigned to 9 individual feeding pens, and then assigned to one of the three dietary treatments according to a completely randomized experimental design. Each treatment group consisted of three pen replicates with one pig per pen.

A corn and soybean meal based diet (Diet 1; a lysine-deficient diet) was formulated to meet or exceed the NRC [1] recommended requirements for various nutrients including crude protein (CP) and essential AAs but not lysine. Diet 2 (a lysine-adequate diet) and Diet 3 (a lysine-excess diet) were formulated by adding L-Lysine Monohydrochloride (Archer Daniels Midland Co., Quincy, IL, USA) to Diet 1 at the expense of corn at rates of 0.35% and 0.70%, respectively (Table 3). The total lysine contents in Diets 1, 2, and 3 were 4.30, 7.10, and 9.80 g/kg (calculated, as-fed basis), respectively.

The feeding trial lasted five weeks, during which pigs were allowed *ad libitum* access to the experimental diets and fresh water. All the pigs, feeders, waterers, and room temperature were

checked 2 to 3 times (6:00 am to 7:00 pm) on a daily basis. At the end of the feeding trial, the pigs were slaughtered in the Meat Science and Muscle Biology Laboratory of Mississippi State University, and muscle samples (approximately 5 g each) were collected from the middle portion of *longissimus dorsi* (between the 10th and 12th ribs) of each pig and snap frozen in liquid nitrogen immediately after the collection. The frozen muscle samples were then transferred to a -80 °C freezer for storage until laboratory analyses of gene expression.

Table 3. Feed ingredients and nutrient composition of experimental diets fed to finishing pigs (as-fed basis) ¹.

	Diet 1	Diet 2	Diet 3
<i>Ingredients (g/kg)</i>			
Corn	908.44	904.94	901.44
Soybean meal	64.00	64.00	64.00
Canola oil	8.00	8.00	8.00
L-Lysine-HCl (98.5%)	0.00	3.50	7.00
DL-Methionine (99.0%)	0.40	0.40	0.40
L-Threonine (98.5%)	0.90	0.90	0.90
L-Tryptophan (99.0%)	0.35	0.35	0.35
Limestone	6.50	6.50	6.50
Dicalcium phosphate	9.00	9.00	9.00
Salt	2.00	2.00	2.00
Mineral premix ²	0.33	0.33	0.33
Vitamin premix ³	0.08	0.08	0.08
Total	1000.0	1000.0	1000.0
<i>Composition (g/kg)⁴</i>			
Metabolizable energy (kcal/kg)	3319	3323	3326
Crude protein	104.5	107.5	110.5
Total lysine	4.33	7.08	9.82
Total methionine	2.37	2.36	2.36
Total threonine	5.02	5.01	5.00
Total tryptophan	1.40	1.40	1.39
Total Ca	4.58	4.58	4.58
Total P	4.32	4.31	4.30

¹ Diets 1, 2, and 3 were formulated to contain total lysine 4.33, 7.08, and 9.82 g/kg (as-fed basis), respectively, of which Diets 2 and 3 were formulated by adding 3.50 and 7.00 g/kg L-lysine-HCl (Archer Daniels Midland Co., Quincy, IL, USA) to Diet 1 at the expense of corn.

² The mineral premix contained Ca 132 g/kg, Cu 10.0 g/kg, Fe 80.0 g/kg, Mn 50.0 g/kg, Zn 100.0 g/kg, I 500 mg/kg, and Se 300 mg/kg.

³ Each kilogram of vitamin premix contained the following: 22.05 million IU vitamin A, 3.31 million IU vitamin D3, 66,138 IU vitamin E, 88 mg vitamin B12, 220 mg biotin, 8,818 mg menadione, 15,432 mg riboflavin, 61,728 mg d-pantothenic acid, and 88,183 mg niacin.

⁴ Calculated major nutrients.

4.2. Preparation of Total RNA

For each muscle sample, the total RNA was extracted from approximately 50 mg of frozen tissue using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, frozen tissue was homogenized in a 15-mL polypropylene centrifuge tube using a Polytron mixer (0.5 mL TRIzol per 50 mg tissue) and the homogenate transferred to a 1.5-mL microcentrifuge tubes. Chloroform (400 μ L/tube) was used to separate RNA from DNA and proteins, and then the total RNA was precipitated with isopropyl alcohol (at 1:1 ratio) and washed with 750 μ L of 75% ethanol. The resulting RNA was air-dried, dissolved in 60 μ L RNase-free water, and stored at -80 °C freezer. The quality of the RNA samples was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the RNA concentrations determined by quantitation using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

4.3. Microarray Analysis

The Affymetrix Porcine Gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA, USA), which contains 394,589 probes representing 19,211 genes, was used to investigate the effect of dietary lysine on the potential change in the skeletal muscle gene expression of pigs. Microarray analysis was conducted at the University of Mississippi Medical Center Molecular and Genomics Core Facility (Jackson, MS, USA) according to the manufacturer's recommended protocol (Affymetrix). Briefly, the Ambion WT Expression Kit (Affymetrix) was used to generate amplified sense-strand cDNA ready for fragmentation and labeling using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). Subsequently, the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) was used to hybridize the gene chips in the GeneChip Hybridization Oven 640, using one chip per RNA sample. After hybridization, the chips were washed and stained on a GeneChip Fluidics Station 450. The reaction image and signals were read with a GeneChip Scanner (GCS 3000, 7G) and data were collected using the GCOS software (version 1.2). The raw gene expression intensity values from the software (i.e., the *.cel files) were imported into PGS software (Partek Inc.) for analysis. For background correction, the algorithm of Robust Multichip Averaging adjusted with probe sequence and GC oligo contents was implemented. The background-corrected data were further converted into expression values using quantile normalization across all the chips and median polish summarization of multiple probes for each probe set [69,70].

All GeneChip transcripts were annotated using the NetAffx annotation database for Exon/Gene on Porcine GeneChip Array, provided online by the manufacturer (<http://www.affymetrix.com/estore/analysis/index.affx>; accessed October 17, 2015). The dietary treatment-induced effects on the expression of all the transcripts were subjected to one-way ANOVA analysis using the PGS software [71]. To achieve a higher degree of confidence, the transcripts showing treatment effects at the significance level of $P \leq 0.01$ were defined as being differentially expressed. These differentially expressed genes or gene transcripts (DEG) were subjected to bioinformatic analysis using the Core Analysis program of the IPA online software (<http://www.ingenuity.com>; Ingenuity Systems, Inc., Redwood City, CA, USA).

4.4. Real-time RT-PCR Analysis

Semi-quantitative real-time reverse-transcribed (RT)-PCR methodology was employed to verify the changes of the mRNA expression levels of nine selected genes, which were PHGDH, PSPH, SCD, CIDEC, AMD1, ZIC1, ERLEC1, DNAJA1 and ZNF181, whose expression levels were found altered ($P \leq 0.01$) from the microarray analysis. These nine DEG were selected because they play critical roles in the metabolic pathways related to muscle growth and development.

First-strand cDNAs were reverse-transcribed from 1 μ g of total RNA by using QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA, USA). The semi-quantitative PCR analysis was performed using the Rotor-Gene Q System and the Rotor-Gene SYBR Green PCR Kit (QIAGEN), followed by melting curve analysis to verify the specificity and identity of the PCR products. The

thermal cycling parameters were 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. Primers for the selected genes were designed by using PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA, USA). The sequences of the designed primers and the relevant information associated with the PCR reactions for these selected genes are shown in Table 4. Also shown in Table 4 is the endogenous control gene, HPRT1 (hypoxanthine phosphoribosyltransferase), used for normalization of any variation during the process of sample preparation [72].

The $\Delta\Delta CT$ method was used for mRNA quantity calculation [73]. Briefly, the raw quantity of a given gene was normalized against the raw quantity of HPRT1 reference gene of a given sample obtained from the Rotor-Gene Q System, and then the normalized level of the given gene of each sample was expressed as a quantity relative to the mean of the normalized quantities of the given gene of the Diet 2 treatment group.

Table 4. The primer pairs used in the semi-quantitative real-time RT-PCR analysis of the selected genes.

Gene symbol ¹	GenBank ²	Sequence (5'–3') ³	Amplicon size (bp)
PHGDH	NM_001123162	F: GCGGTTTGGTTTAGGTGTTTC R: AAGGGTCCAGGCTATCACT	113
PSPH	NM_001243221	F: CTGCAGGCTCCAGTTTAGTT R: CTCGCAGAGTCTTTACCAACA	97
SCD	NM_213781	F: CCCAAGGCAGACAAGAGAATAG R: GTGTTGACGACTGAGGTTACAG	91
CIDEA	NM_001112689	F: CCAACTCTCCCTCTCCCATAA R: CATGTTCAAGCAACCAATGAAG	106
AMD1	XM_003121345	F: TCCACAAGTCAAGTCCTCTAATG R: CCATGGAGAGGAACGAATCAA	108
ZIC1	XM_003358599	F: CGACCGACGCTTTGCTAATA R: GTAGGACTTGTCGCACATCTT	97
ERLEC1	XM_003125147	F: GCTGGCTATCCTTTGTACTCTC R: CAACACTGCTTGTGGACATTT	109
DNAJA1	NM_001244163	F: GGTGGTAAGAAAGGAGCAGTAG R: CTGAACCATTCCAGGTCCTATT	93
ZNF181	NM_001244818	F: GCCTTCAGCCAAAGCAAATC R: AGGCTTTCCACATTCCTACTAC	85
HPRT1 ⁴	NM_001032376	F: GCTATGCCCTTGACTACAATGA R: TTGAACCTCTCTTAGGCTTTG	102

¹ PHGDH = Phosphoglycerate dehydrogenase; PSPH = Phosphoserine phosphatase; SCD = Stearoyl-CoA desaturase; CIDEA = Cell death-inducing DFFA-like effector c; AMD1 = Adenosylmethionine decarboxylase 1; ZIC1 = Zic family member 1; ERLEC1 = Endoplasmic reticulum lectin 1; DNAJA1 = DnaJ (Hsp40) homolog, subfamily A, member 1; ZNF181 = Zinc finger protein 181; HPRT1 = Hypoxanthine phosphoribosyltransferase 1.

² The accession number of the cDNA sequences retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov>) for primer design.

³ F = a forward primer, and R = a reverse primer.

⁴ HPRT1 was selected as an internal control reference gene for the purpose of normalization of the expression of other gene.

4.5. Statistical Analysis

Dietary treatment effects on the relative mRNA expression levels, obtained from the microarray analysis for each gene transcript, were subjected to analysis of variance (ANOVA) for a completely randomized experimental design using the PGS software. For a higher degree of confidence, when the ANOVA test gave a *P* value less than or equal to 0.01, the treatment means were compared by two orthogonal contrasts, which were Diet 1 vs. Diet 2 and Diet 3 vs. Diet 2. Since this project was simply an animal nutrition study, no animals were sick and there were not any clinical differences in terms of animal health status observed among the three treatments. So not many genes were expected to be altered by the lysine levels studied with respect to their mRNA expression. Therefore, the statistical procedure of False Discovery Rate that is usually used in animal disease models to control the false positives among the large numbers of significant positives was not employed in this study [69,74].

In addition, the dietary treatment effects on the relative expression levels of the selected DEG analyzed by real-time RT-PCR were also subjected to ANOVA and orthogonal contrast analyses (Diet 1 vs. Diet 2, and Diet 3 vs. Diet 2) using the General Linear Model procedure of SAS (SAS 9.4; SAS Institute Inc., Cary, NC, USA). When the contrast *P* values were less than or equal to 0.05, the comparison was considered to have a significant difference. For the canonical pathway analysis with IPA, the *P* values less than or equal to 0.05 or -log of *P*-value greater than or equal to 1.3 were considered significant.

5. Conclusions

Dietary lysine affected the expression levels of at least 131 gene transcripts in the *longissimus dorsi* muscle of the late-stage finishing pigs, of which 60 transcripts belonging to 59 genes were annotated in the NetAffx database. Dietary lysine deficiency may lead to (1) increased muscle protein degradation via the ubiquitination pathway as indicated by the up-regulated DNAJA1, HSP90AB1 and UBE2B mRNA levels, (2) reduced muscle protein synthesis via the upregulation of RND3 and ZIC1 mRNA levels, (3) increased serine and glycine synthesis in the muscle as indicated by the increased PHGDH and PSPH mRNA levels, and (4) increased lipid accumulation via the increased expression of ME1, SCD, and CIDEC mRNA levels. Dietary lysine excess may lead to (1) decreased muscle protein degradation via the downregulation of mRNA levels of DNAJA1, HSP90AA1, HSPH1, and UBE2D2, and (2) reduced lipid biosynthesis via the decreased expression of CFD and ME1 mRNA in porcine skeletal muscle.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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Author Contributions: S.F.L. conceived and designed the experiments; J.R.B. provided the research resources; T.W. performed the experiments with substantial help from N.R. in the animal trial and sample collection part; M.A.C. guided T.W. and N.R. in the animal trial, and J.M.F. guided T.W. in laboratory work and data analysis; T.W. and S.F.L. analyzed the data and wrote the paper. All authors contributed to the final editing of the manuscript.

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Abbreviations

AA	Amino acid
ADG	Average daily gain
ANOVA	Analysis of variance
CP	Crude protein
CT	Threshold cycle
DEG	Differentially expressed genes or gene transcripts
ER	Endoplasmic reticulum
GC	Guanine-cytosine
GEO	Gene Expression Omnibus
GCOS	GeneChip Operating Software
IPA	Ingenuity Pathways Analysis
MIAMIE	Minimum information about a microarray experiment
NIFA	National Institute of Food and Agriculture
NRC	National Research Council
PGS	Partek Genomics Suite
PM	Plasma membrane
RT	Reverse transcription
SAS	Statistical Analysis System
USDA	U.S. Department of Agriculture

References

1. NRC. Proteins and amino acids. In *Nutrient Requirements of Swine*, 11th rev. ed.; The National Academies Press: Washington, DC, USA, **2012**; pp 15-44.
2. Austin, J.L. Amino acids in swine nutrition. In *Swine Nutrition*, 2nd ed.; Lewis, A.J., Southern, L.L. Eds.; CRC Press: Boca Raton, FL, USA, **2001**; pp 131-150.
3. Roy, N.; Lapierre, H.; Bernier J.F. Whole-body protein metabolism and plasma profiles of amino acids and hormones in growing barrows fed diets adequate or deficient in lysine. *Can. J. Anim. Sci.* **2000**, *80*, 585-595.
4. Shelton, N.W.; Tokach, M.D.; Dritz, S.S.; Goodband, R.D.; Nelssen, J.L.; DeRouchey, J.M. Effects of increasing dietary standardized ileal digestible lysine for gilts grown in a commercial finishing environment. *J. Anim. Sci.* **2011**, *89*, 3587-3595.
5. Witte, D.P.; Ellis, M.; McKeith, F.K.; Wilson, E.R. Effect of dietary lysine level and environmental temperature during the finishing phase on the intramuscular fat content of pork. *J. Anim. Sci.* **2000**, *78*, 1272-1276.
6. Bidner, B.S.; Ellis, M.; Witte, D.P.; Carr, S.N.; McKeith, F.K. Influence of dietary lysine level, pre-slaughter fasting, and rendement napole genotype on fresh pork quality. *Meat Sci.* **2004**, *68*, 53-60.
7. Tous, N.; Lizardo, R.; Vila, B.; Gispert, M.; Font, I.F.M.; Esteve-Garcia, E. Effect of reducing dietary protein and lysine on growth performance, carcass characteristics, intramuscular fat, and fatty acid profile of finishing barrows. *J. Anim. Sci.* **2014**, *92*, 129-140.
8. Jefferson, L.S.; Kimball, S.R. Amino acid regulation of gene expression. *J. Nutr.* **2001**, *131*, 2460S-2466S.
9. Crozier, S.J.; Kimball, S.R.; Emmert, S.W.; Anthony, J.C.; Jefferson, L.S. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J. Nutr.* **2005**, *135*, 376-382.
10. Yao, K.; Yin, Y.L.; Chu, W.; Liu, Z.; Deng, D.; Li, T.; Huang, R.; Zhang, J.; Tan, B.; Wang, W.; Wu, G. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. *J. Nutr.* **2008**, *138*, 867-872.
11. Bonetto, A.; Penna, F.; Minero, V.G.; Reffo, P.; Costamagna, D.; Bonelli, G.; Baccino, F.M.; Costelli, P. Glutamine prevents myostatin hyperexpression and protein hypercatabolism induced in C2C12 myotubes by tumor necrosis factor-alpha. *Amino acids* **2011**, *40*, 585-594.
12. Prada, P.O.; Hirabara, S.M.; de Souza, C.T.; Schenka, A.A.; Zecchin, H.G.; Vassallo, J.; Velloso, L.A.; Carneiro, E.; Carvalheira, J.B.; Curi, R.; Saad, M.J. L-glutamine supplementation induces insulin resistance

- in adipose tissue and improves insulin signalling in liver and muscle of rats with diet-induced obesity. *Diabetologia* **2007**, *50*, 1949-1959.
13. Vinciguerra, M.; Musaro, A.; Rosenthal, N. Regulation of muscle atrophy in aging and disease. *Adv. Exp. Med. Biol.* **2010**, *694*, 211-233.
 14. Ruegg, M.A.; Glass, D.J. Molecular mechanisms and treatment options for muscle wasting diseases. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*: 373-95.
 15. Bonaldo, P.; Sandri, M. Cellular and molecular mechanisms of muscle atrophy. *Dis. Model. Mech.* **2013**, *6*: 25-39.
 16. Miller, E.R.; Ullrey, D.E. The pig as a model for human-nutrition. *Annu. Rev. Nutr.* **1987**, *7*: 361-382.
 17. Guilloteau, P.; Zabielski, R.; Hammon, H.M.; Metges, C.C. Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? *Nutr. Res. Rev.* **2010**, *23*: 4-22.
 18. Swindle, M.M.; Makin, A.; Herron, A.J.; Clubb, F.J.; Frazier, K.S. Swine as models in biomedical research and toxicology testing. *Vet. Pathol.* **2012**, *49*: 344-356.
 19. Wang, T.; Crenshaw, M.A.; Regmi, N.; Armstrong, T.; Blanton, J.R.; Liao, S.F. Effect of dietary lysine fed to pigs at late finishing stage on market-value associated carcass characteristics. *J. Anim. Vet. Adv.* **2015**, *14*, 232-236.
 20. Brazma, A.; Hingamp, P.; Quackenbush, J.; Sherlock, G.; Spellman, P.; Stoeckert, C.; Aach, J.; Ansorge, W.; Ball, C.A.; Causton, H.C.; Gaasterland, T.; Glenisson, P.; Holstege, F.C.P.; Kim, I.F.; Markowitz, V.; Matese, J.C.; Parkinson, H.; Robinson, A.; Sarkans, U.; Schulze-Kremer, S.; Stewart, J.; Taylor, R.; Vilo, J.; Vingron, M. Minimum information about a microarray experiment (MIAME) toward standards for microarray data. *Nat. Genet.* **2001**, *29*, 365-371.
 21. Fafournoux, P.; Bruhat, A.; Jousse, C. Amino acid regulation of gene expression. *Biochem. J.* **2000**, *351*, 1-12.
 22. Ishida, A.; Kyoya, T.; Nakashima, K.; Katsumata, M. Muscle protein metabolism during compensatory growth with changing dietary lysine levels from deficient to sufficient in growing rats. *J. Nutr. Sci. Vitaminol.* **2011**, *57*, 401-408.
 23. Richard, I.; Broux, O.; Allamand, V.; Fougereousse, F.; Chiannilkulchai, N.; Bourg, N.; Brenguier, L.; Devaud, C.; Pasturaud, P.; Roudaut, C.; Hillaire, D.; Passos-Bueno, M.-R.; Zatz, M.; Tischfield, J.A.; Fardeau, M.; Jackson, C.E.; Cohen, D.; Beckmann, J.S. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **1995**, *81*, 27-40.
 24. Fujimori, T.; Kamiya, Y.; Nagata, K.; Kato, K.; Hosokawa, N. Endoplasmic reticulum lectin XTP3-B inhibits endoplasmic reticulum-associated degradation of a misfolded alpha1-antitrypsin variant. *FEBS J.* **2013**, *280*, 1563-1575.
 25. Bernard, C.; Cassar-Malek, I.; Le Cunff, M.; Dubroeucq, H.; Renand, G.; Hocquette, J.F. New indicators of beef sensory quality revealed by expression of specific genes. *J. Agric. Food Chem.* **2007**, *55*, 5229-5237.
 26. Chardin, P. Function and regulation of Rnd proteins. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 54-62.
 27. Zhao, H.; Yang, J.; Fan, T.; Li, S.; Ren, X. RhoE functions as a tumor suppressor in esophageal squamous cell carcinoma and modulates the PTEN/PI3K/Akt signaling pathway. *Tumour Biol.* **2012**, *33*, 1363-1374.
 28. Merzdorf, C.S.; Sive, H.L. The *zic1* gene is an activator of Wnt signaling. *Int. J. Dev. Biol.* **2006**, *50*, 611-617.
 29. Zhong, J.; Chen, S.; Xue, M.; Du, Q.; Cai, J.; Jin, H.; Si, J.; Wang, L. ZIC1 modulates cell-cycle distributions and cell migration through regulation of sonic hedgehog, PI(3)K and MAPK signaling pathways in gastric cancer. *BMC Cancer* **2012**, *12*, 290-299.
 30. Dennis, P.B.; Fumagalli, S.; Thomas, G. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Dev.* **1999**, *9*, 49-54.
 31. Sato, T.; Ito, Y.; Nedachi, T.; Nagasawa, T. Lysine suppresses protein degradation through autophagic-lysosomal system in C2C12 myotubes. *Mol. Cell Biochem.* **2014**, *391*, 37-46.
 32. Wu, G. Synthesis of amino acids. In *Amino Acids: Biochemistry and Nutrition*; 1st ed.; CRC Press: Boca Raton, FL, USA, **2013**; pp 63-96.
 33. Regmi, N.; Wang, T.; Crenshaw, M.A.; Rude, B.J.; Wu, G.; Liao, S.F. Effects of dietary levels on plasma free amino acid profile in late-stage finishing pigs. *SpringerPlus.* **2016**, *5*, 888 (1-9).
 34. Wu, G. Physiological functions of amino acids. In *Amino Acids: Biochemistry and Nutrition*; CRC Press: Boca Raton, FL, USA, **2013**; pp 339-390.
 35. Lee, N.K.L.; MacLean, H.E. Polyamines, androgens, and skeletal muscle hypertrophy. *J. Cell Physio.* **2011**, *226*, 1453-1460.

36. von Deutsch, D.A.; Abukhalaf, I.K.; Wineski, L.E.; Silvestrov, N.A.; Bayorh, M.A.; Potter, D.E. Changes in muscle proteins and spermidine content in response to unloading and clenbuterol treatment. *Can. J. Physiol. Pharmacol.* **2003**, *81*, 28-39.
37. Wu, G.; Morris, S.M., Jr. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* **1998**, *336*, 1-17.
38. Vidal, O.; Varona, L.; Oliver, M.A.; Noguera, J.L.; Sanchez, A.; Amills, M. Malic enzyme 1 genotype is associated with backfat thickness and meat quality traits in pigs. *Anim. Genet.* **2006**, *37*, 28-32.
39. Mourot, J.; Kouba, M. Development of intra- and intermuscular adipose tissue in growing large white and Meishan pigs. *Reprod. Nutr. Dev.* **1999**, *39*, 125-132.
40. Ntambi, J.M.; Miyazaki, M. Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog. Lipid Res.* **2004**, *43*, 91-104.
41. Bessa, R.J.; Hughes, R.A.; Jeronimo, E.; Moreira, O.C.; Prates, J.A.; Doran, O. Effect of pig breed and dietary protein level on selected fatty acids and stearoyl-coenzyme A desaturase protein expression in longissimus muscle and subcutaneous fat. *J. Anim. Sci.* **2013**, *91*, 4540-4546.
42. da Costa, N.; McGillivray, C.; Bai, Q.; Wood, J.D.; Evans, G.; Chang, K.-C. Restriction of dietary energy and protein induces molecular changes in young porcine skeletal muscles. *J. Nutr.* **2004**, *134*, 2191-2199.
43. Vilà-Brau, A.; De Sousa-Coelho, A.L.; Gonçalves, J.F.; Haro, D.; Marrero, P.F. Fsp27/CIDEA is a CREB target gene induced during early fasting in liver and regulated by FA oxidation rate. *J. Lipid Res.* **2013**, *54*, 592-601.
44. Ronti, T.; Lupattelli, G.; Mannarino, E. The endocrine function of adipose tissue: an update. *Clin. Endocrinol.* **2006**, *64*, 355-365.
45. Lan, H.; Rabaglia, M.E.; Stoeck, J.P.; Nadler, S.T.; Schueler, K.L.; Zou, F.; Yandell, B.S.; Attie, A.D. Gene expression profiles of nondiabetic and diabetic obese mice suggest a role of hepatic lipogenic capacity in diabetes susceptibility. *Diabetes* **2003**, *52*, 688-700.
46. Regmi, N.; Wang, T.; Crenshaw, M.A.; Rude, B.J.; Liao, S.F. Limiting dietary lysine increased plasma concentration of total cholesterol in finishing pigs. *J. Anim. Sci.* **2015**, *93*(Suppl. s3), 66.
47. Inuzuka, M.; Hayakawa, M.; Ingi, T. Serinc, an activity-regulated protein family, incorporates serine into membrane lipid synthesis. *J. Biol. Chem.* **2005**, *280*, 35776-35783.
48. Schauder, C.M.; Wu, X.; Saheki, Y.; Narayanaswamy, P.; Torta, F.; Wenk, M.R.; De Camilli, P.; Reinisch, K.M. Structure of a lipid-bound extended synaptotagmin indicates a role in lipid transfer. *Nature* **2014**, *510*, 552-555.
49. Chang, C.L.; Hsieh, T.S.; Yang, T.T.; Rothberg, K.; Azizoglu, D.B.; Volk, E.; Liao, J.C.; Liou, J. Feedback regulation of receptor-induced Ca²⁺ signaling mediated by E-Syt1 and Nir2 at endoplasmic reticulum-plasma membrane junctions. *Cell Rep.* **2013**, *5*, 813-825.
50. Civitelli, R.; Villareal, D.T.; Agnusdei, D.; Nardi, P.; Avioli, L.V.; Gennari, C. Dietary L-lysine and calcium metabolism in humans. *Nutrition* **1992**, *8*, 400-405.
51. Solberg, A.; Robertson, A.B.; Aronsen, J.M.; Rognmo, O.; Sjaastad, I.; Wisloff, U.; Klungland, A. Deletion of mouse Alkbh7 leads to obesity. *J. Mol. Cell Biol.* **2013**, *5*, 194-203.
52. Than, N.G.; Sumegi, B.; Than, G.N.; Berente, Z.; Bohn, H. Isolation and sequence analysis of a cDNA encoding human placental tissue protein 13 (PP13), a new lysophospholipase, homologue of human eosinophil Charcot-Leyden Crystal protein. *Placenta* **1999**, *20*, 703-710.
53. Li, J.; Romestaing, C.; Han, X.; Li, Y.; Hao, X.; Wu, Y.; Sun, C.; Liu, X.; Jefferson, L.S.; Xiong, J.; Lanoue, K.F.; Chang, Z.; Lynch, C.J.; Wang, H.; Shi, Y. Cardiolipin remodeling by ALCAT1 links oxidative stress and mitochondrial dysfunction to obesity. *Cell Metab.* **2010**, *12*, 154-165.
54. Larsen, R.D.; Ernst, L.K.; Nair, R.P.; Lowe, J.B. Molecular cloning, sequence, and expression of a human GDP-L-fucose:beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen. *PNAS* **1990**, *87*, 6674-6678.
55. Wan, D.; Gong, Y.; Qin, W.; Zhang, P.; Li, J.; Wei, L.; Zhou, X.; Li, H.; Qiu, X.; Zhong, F.; He, L.; Yu, J.; Yao, G.; Jiang, H.; Qian, L.; Yu, Y.; Shu, H.; Chen, X.; Xu, H.; Guo, M.; Pan, Z.; Chen, Y.; Ge, C.; Yang, S.; Gu, J. Large-scale cDNA transfection screening for genes related to cancer development and progression. *PNAS* **2004**, *101*, 15724-15729.
56. Gong, F.; Peng, X.; Sang, Y.; Qiu, M.; Luo, C.; He, Z.; Zhao, X.; Tong, A. Dichloroacetate induces protective autophagy in LoVo cells: involvement of cathepsin D/thioredoxin-like protein 1 and Akt-mTOR-mediated signaling. *Cell Death Dis.* **2013**, *4*, e913.

57. Takahashi, S.; Ebihara, A.; Kajiho, H.; Kontani, K.; Nishina, H.; Katada, T. RASSF7 negatively regulates pro-apoptotic JNK signaling by inhibiting the activity of phosphorylated-MKK7. *Cell Death Differ.* **2011**, *18*, 645-655.
58. Weston, C.R.; Davis, R.J. The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.* **2002**, *12*, 14-21.
59. Recino, A.; Sherwood, V.; Flaxman, A.; Cooper, W.N.; Latif, F.; Ward, A.; Chalmers, A.D. Human RASSF7 regulates the microtubule cytoskeleton and is required for spindle formation, Aurora B activation and chromosomal congression during mitosis. *Biochem. J.* **2010**, *430*, 207-213.
60. Praetorius, J.; Andreasen, D.; Jensen, B.L.; Ainsworth, M.A.; Friis, U.G.; Johansen, T. NHE1, NHE2, and NHE3 contribute to regulation of intracellular pH in murine duodenal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2000**, *278*, G197-206.
61. Zou, C.; Li, J.; Bai, Y.; Gunning, W.T.; Wazer, D.E.; Band, V.; Gao, Q. Centrobin: a novel daughter centriole-associated protein that is required for centriole duplication. *J. Cell Biol.* **2005**, *171*, 437-445.
62. Savage, J.R.; McLaughlin, J.N.; Skiba, N.P.; Hamm, H.E.; Willardson, B.M. Functional roles of the two domains of phosducin and phosducin-like protein. *J. Biol. Chem.* **2000**, *275*, 30399-30407.
63. Gong, K.Q.; Yallowitz, A.R.; Sun, H.; Dressler, G.R.; Wellik, D.M. A Hox-Eya-Pax complex regulates early kidney developmental gene expression. *Mol. Cell Biol.* **2007**, *27*, 7661-7668.
64. Begum, M.; Tashiro, H.; Katabuchi, H.; Suzuki, A.; Kurman, R.J.; Okamura, H. Neonatal estrogenic exposure suppresses PTEN-related endometrial carcinogenesis in recombinant mice. *Lab invest.* **2006**, *86*, 286-296.
65. Laity, J.H.; Lee, B.M.; Wright, P.E. Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol.* **2001**, *11*, 39-46.
66. Tominaga, K.; Matzuk, M.M.; Pereira-Smith, O.M. MrgX is not essential for cell growth and development in the mouse. *Mol. Cell Biol.* **2005**, *25*, 4873-4880.
67. Suzuki, F.; Goto, M.; Sawa, C.; Ito, S.; Watanabe, H.; Sawada, J.; Handa, H. Functional interactions of transcription factor human GA-binding protein subunits. *J. Biol. Chem.* **1998**, *273*, 29302-29308.
68. Chen, P.J.; Huang, Y.S. CPEB2-eEF2 interaction impedes HIF-1 α RNA translation. *EMBO J.* **2012**, *31*, 959-971.
69. Liao, S.F.; Brown, K.R.; Stromberg, A.J.; Burris, W.R.; Boling, J.A.; Matthews, J.C. Dietary supplementation of selenium in inorganic and organic forms differentially and commonly alters blood and liver selenium concentrations and liver gene expression profiles of growing beef heifers. *Biol. Trace Elem. Res.* **2011**, *140*, 151-169.
70. Liao, S.F.; Boling, J.A.; Matthews, J.C. Gene expression profiling indicates an increased capacity for proline, serine, and ATP synthesis and mitochondrial mass by the liver of steers grazing high vs. low endophyte-infected tall fescue. *J. Anim. Sci.* **2015**, *93*, 5659-5671.
71. Partek Inc. Partek documentation: Turning data into discovery. St. Louis, MO, USA, **2009**. pp. 1-498.
72. Nygard, A.-B.; Jørgensen, C.B.; Cirera, S.; Fredholm, M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol. Bio.* **2007**, *8*, 67-72.
73. Applied Biosystems. The Comparative Ct Method ($\Delta\Delta$ CT Method). In *Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR*. Foster City, CA, USA, **2008**. pp 52-59.
74. Allison, D.B.; Cui, X.; Page, G.P.; Sabripour, M. Microarray data analysis: from disarray to consolidation and consensus. *Nat. Rev. Genet.* **2006**, *7*, 55-65.

