DIFFERENTIAL GENE EXPRESSION AND MOLECULAR MECHANISMS ASSOCIATED WITH DEVELOPMENT OF PALE, SOFT AND EXUDATIVE (PSE) TURKEY MEAT

By

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ABSTRACT

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The success of turkey breeding has coincided with an increased incidence of a meat quality defect known as pale, soft and exudative (PSE) meat. Application of molecular-based approaches such as genetic markers for animal selection or pathway intervention to prevent development of this meat defect have been suggested as a potential long-term solution. However, molecular mechanisms associated with this alteration remain unclear. The overall goal of this study was to obtain better understanding of molecular mechanisms underlying development of PSE turkey. The study comprised two specific aims: 1) to assess global differential gene expression between normal and PSE turkey; and 2) to confirm differences between normal and PSE meat samples at the protein level of a candidate gene selected from aim 1. Turkey breast muscle samples were collected from 22wk randombred control line (RBC2) and 16wk commercial (COMM) turkeys. Breast samples were classified as normal or PSE based on marinade uptake (high = normal, low = PSE). Total RNA was isolated from muscle samples with the highest (normal, n = 6) and the lowest (PSE, n = 6) marinade uptake. Transcriptome analyses were conducted using two platforms: the turkey skeletal muscle long oligonucleotide microarray, and deep transcriptome sequencing with an Illumina Genome Analyzer IIX (RNA-Seq). The microarray study of RBC2 samples revealed

49 differentially expressed transcripts (false discovery rate, FDR < 0.1). Genes selected for pathway analysis were determined using two criteria: fold change ranking (FC < -1.66, FC > 1.66) and FDR < 0.35. The calcium signaling pathway was highlighted as the top canonical pathway. In addition, changes in expression of genes in the actin cytoskeleton signaling pathway suggested altered structures of actin filaments that may affect strength and flexibility of muscle cells. In RNA-Seq analysis, four RNA samples for each of the extreme normal and PSE characteristics from the RBC2 line were sequenced (n = 4). Pathway analysis of 494 differentially expressed transcripts (FDR < 0.05) identified by RNA-Seq confirmed previously suggested changes in calcium homeostasis and organization of actin cytoskeleton. Pyruvate dehydrogenase kinase isozyme 4 (PDK4), which regulates glucose oxidation, showed substantial decreased expression with both microarray (FC = -25.9) and RNA-Seq (FC = -14.1); thus, this gene was chosen as a candidate gene for further evaluation. The protein abundance of PDK4 was significantly decreased (FC = -3.4, P < 0.001) in PSE samples (n = 6) of the RBC2 line. Reduced expression of PDK4 at both transcriptional (FC = -12.8, P < 0.05) and translational levels (FC = -2.8, P < 0.001) was also observed in PSE turkey of the COMM line (n = 6), supporting the biological relevance of PDK4 suppression in the development of PSE turkey, and also suggesting that the mechanism responsible for the decreased PDK4 in RBC2 turkey subpopulations has been maintained in a commercial line. By identifying several candidate genes including PDK4, this study lays the foundation for future studies aimed at defining the mechanisms of development of PSE turkey.

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CHAPTER 1

INTRODUCTION

For the past several decades, the meat consumption pattern in the US has shifted from predominantly red meat to white meat, as many consumers prefer meat and meat products that are low in both fat and calories. This trend is evidenced by the decrease in average annual consumption of red meat (beef and pork) from 134 lb per person in 1965 to 104 lb in 2011, and the increase in consumption of poultry from 40 lb per person to 100 lb in the same period of time (US Department of Agriculture, 2013).

Turkey breast meat has become a preferred source of white meat. Due to its low fat content and high quality protein as well as its relatively inexpensive price, turkey and its processed products have become common daily food choices of consumers. In the US alone, average per capita annual consumption of turkey has increased from 8.7 lbs in 1978 to 15.7 lbs in 1988, and has remained steadily high at approximate 16 lbs from 1989 to 2012 (US Department of Agriculture, 2013).

In response to an increase in consumer demand, turkey production has steadily risen. According to the Food and Agriculture Organization of the United Nations (2013), 1.5 billion turkeys were raised, yielding 32 billion lbs meat during the 1970s, but in the 2000s, global production of turkeys rose to 4.2 billion live birds producing 107 billion lbs of turkey meat. To meet this growing demand, turkeys have been intensively selected for rapid growth rate, large breast muscle mass and reduced abdominal fat. However, the success of breeding has coincided with an

increase in prevalence of a meat defect, described as pale, soft, and exudative (**PSE**) meat in turkeys (Dransfield and Sosnicki, 1999; Owens et al., 2000).

The term PSE was originally used to describe pork with discoloration and poor water holding capacity (reviewed by Cassens, 2000). It is well recognized that development of both PSE pork and poultry are associated with rapid early-stage postmortem metabolism (Pietrizak et al., 1997; Le Bihan-Duval et al., 2003), leading to excessive lactic acid accumulation in the muscle while the carcass is still warm. The combination of high early postmortem acidity and heat initiates extensive denaturation of proteins; as a consequence, muscle fibers shrink and expel water in the form of drip that contributes to soft and flaccid texture of the PSE meat. The loss of myoglobin, the water-soluble red pigment in muscle, as exudate, causes the pale appearance.

Denatured myofibrillar proteins present poor protein functionality characteristics, particularly water holding capacity and protein solubility. Such conditions detrimentally affect the quality of products from PSE meat. Packaged cuts surrounded by exudate are unappealing to customers. Most turkey is processed into value-added products and it is with these products that the PSE problem presents the greatest challenge. Because PSE meat possesses poor protein solubility properties, myofibrillar proteins are inadequately extracted during the manufacturing process, resulting in products with reduced cohesiveness and inconsistent texture (reviewed by Solomon et al., 1998). In addition to high cook loss resulting from low water holding capacity, products made from PSE meat exhibit low processing yield (Barbut et al., 2005; Bianchi et al., 2005). This defective product does not harmfully

affect consumer health, but it contains reduced level of water-soluble vitamins and minerals, and receives decreased consumer preference (Droval et al., 2012). Overall, the occurrence of PSE meat is economically troublesome for meat producers and processers.

For the turkey industry, the problem of PSE meat has been a great challenge. In the US, PSE incidence causes an average $2 million loss per year to the processors (Owens et al., 2009). The prevalence of PSE turkey is a broad spectrum, but even within a single flock, incidence varies from 5-40% (Owens et al., 2000; Petracci et al., 2009; Zhu et al., 2012). Moreover, even small amounts of PSE meat as raw ingredients in a mixture undergoing processing can significantly affect quality of the final products.

Several attempts have been made to improve the quality of meat products made from PSE turkey by changes in turkey management, through modification of transportation systems, and by processing interventions (reviewed by Barbut et al., 2008). However, these changes in practices have met with only moderate success. The use of food additives that increase water holding capacity and protein binding are promising but this approach generates more cost to the processers, and often does not yield an optimal product. An alternative strategy is to define the underlying mechanisms that make birds become susceptible to PSE development and exploit genetic markers associated with abnormalities in these pathways to select against PSE susceptibility. However, the molecular pathways underlying development of PSE turkey are still not completely understood.

Previous molecular studies regarding development of PSE turkey were developed based on similarities in accelerated early-stage postmortem metabolism in porcine and poultry PSE meat. It has been demonstrated that, in swine, the rapid postmortem metabolism is associated with a leak of the ryanodine receptor (**RYR**), the sarcoplasmic reticulum (**SR**) calcium channel protein, due to a single point mutation of *RYR1* that causes [Ca^{2+}] overload inside the muscle cells (Mickelson et al., 1986; Mickelson et al., 1988a; Fujii et al., 1991; Otsu et al., 1994). However, in turkey, no mutation of *RYR* has yet been found (Chiang et al., 2007). Additionally, although a commercial DNA test for detecting *RYR* mutation in pigs has been developed and implemented, this test accounted for only 25-35% of PSE pork in commercial slaughter houses (reviewed by Barbut et al., 2008). This evidence suggests that development of the PSE phenotype is a multifactorial genetic problem involving not only *RYR*, but possibly many genes in multiple pathways.

The long-term goal of this study is to minimize the occurrence of PSE turkey meat by using molecular-based knowledge to develop pathway intervention approaches. To accomplish this goal, complete understanding of molecular mechanisms associated with development of this turkey meat defect is required. *The central hypothesis for this study is that there are differences at the molecular level between PSE and normal turkey providing complex interactions between intracellular Ca^{2+} homeostasis and other metabolic pathways that lead to meat quality defects.* To test the central hypothesis, the following specific aims are pursued.

Specific Aim 1: ***To identify global differences in transcript abundance between turkey breast muscle samples of birds classified as normal or PSE meat***

Previous studies focusing only on *RYR* were not able to explain development of the turkey PSE phenotype. The working hypothesis of this aim is that development of PSE turkey is associated with changes in expression of multiple genes responsible for numerous molecular pathways.

Under this aim, global gene expression in breast muscle between normal and PSE turkey will be determined using two transcriptome analyses, 1) the turkey skeletal muscle long oligonucleotide (**TSKMLO**) microarray, and 2) the deep transcriptome sequencing technique (**RNA-Seq).**

Transcriptome profiling techniques have been developed for simultaneous investigation of the expression of thousands of genes. Genes that are differentially expressed between treatments can then be clustered into networks based on their biological functions, which aids in identification of candidate pathways associated with the PSE phenotype. Each platform offers different advantages and disadvantages. The microarray method allows direct comparison of global gene expression between two treatments and reports the results as relative expression. The microarray is a well-established transcriptome profiling technique that is relatively inexpensive compared to RNA-Seq; thus, the microarray method allows us to conduct experiments with a larger number of biological replicates, that is more representative of the turkey population. In addition, the TSKMLO microarray, comprising 6000 70-mer oligonucleotides representing turkey skeletal muscle genes

(Sporer et al., 2011), is able to reveal differential expression of genes specific to turkey skeletal muscle. However, in recent years, microarray technology has been gradually superseded by RNA-Seq. Unlike hybridization-based microarray, RNA-Seq has the capability of quantifying actual mRNA abundance of the sample and determining genes beyond selected probes on the microarray (Roh et al., 2010). This platform also provides powerful dynamic range (Hitzemann et al., 2013) with high reproducibility and small technical variation (Marioni et al., 2008). Utilization of both techniques provides complementary results and facilitates greater understanding for development of PSE turkey.

Specific Aim *2*: *To analyze abundance of a candidate protein encoded by a differentially expressed gene with the most biological relevance to development of PSE turkey*

The mRNA abundance determined by transcriptome analysis suggests changes in gene expression in response to biological stimuli and generally correlates with the proportion of encoded protein; however, this is not always the case (Preiss et al., 2003). For this aim, the working hypothesis is that protein abundance of a candidate gene, identified in aim 1, changes in the same direction with transcript difference.

To accomplish this aim, a gene showing significant difference in expression between normal and PSE turkey from aim 1 will be selected as a candidate for determining protein abundance using protein immunoblot assay. The differentially expressed gene with the most apparent biological relevance to the

development of PSE meat will receive the highest priority for protein quantification in this aim.

It is anticipated that results of this study will increase fundamental understanding of molecular mechanisms and pathways altered in PSE-susceptible turkeys resulting in reduced meat quality. Such information is foundational to identification of genetic markers for breeding selection and development of new intervention strategies to prevent development of PSE meat in susceptible birds.

CHAPTER 2

DIFFERENTIAL GENE EXPRESSION BETWEEN NORMAL AND PALE, SOFT AND EXUDATIVE TURKEY MEAT IDENTIFIED BY MICROARRAY ANALYSIS

ABSTRACT

In response to high consumer demand, turkeys have been intensively selected for rapid growth rate, and breast muscle mass and conformation. The success in breeding selection has coincided with an increasing incidence of pale, soft and exudative (PSE) meat defect, especially in response to heat stress. We hypothesized that the underlying mechanism responsible for the development of PSE meat arises from differences in expression of several critical genes. The objective of this study was to determine differential gene expression between normal and PSE turkey meat using a 6K turkey skeletal muscle long oligonucleotide microarray. Turkey breast meat samples were collected from the randombred control line at 22 weeks of age, and classified as normal or PSE primarily based on marinade uptake (high = normal, low = PSE). Total RNA was isolated from meat samples with the highest (normal, n = 6) and the lowest (PSE, n = 6) marinade uptake. Microarray data confirmation was conducted using quantitative real-time PCR. Selection of differentially expressed genes for pathway analysis was performed using a combination of fold change ranking (FC < -1.66, FC > 1.66) and false discovery rate (FDR < 0.35) as criteria. The calcium signaling pathway was highlighted as the top canonical pathway associated with differential gene expression between normal and PSE turkey. Dramatic down-regulation of fast-twitch myosin heavy chain coupled with up-regulation of slow-twitch myosin and troponin C suggested a switch of skeletal muscle isoforms, which may

alter muscle fiber arrangement and formation of actin-myosin complexes. Changes in expression of genes in the actin cytoskeleton signaling pathway also suggest altered structures of actin filaments that may affect cell motility as well as strength and flexibility of muscle cells. Substantial down-regulation of pyruvate dehydrogenase kinase, isozyme 4 was observed in PSE samples suggesting altered regulation of the aerobic metabolic pathway in the birds that developed PSE meat defect.

2.1 Background

Consumer demand for inexpensive food with low fat and high protein content has led to a tremendous growth of the poultry industry over the past several decades. Turkeys have become an attractive protein source because of their larger portion of lean meat compared to chicken. To meet increasing demand for turkey meat, birds have been intensively selected for rapid growth rate and breast muscle mass accretion and conformation (Barbut et al., 2008). However, the success of breeding has coincided with an increase in the prevalence of a significant quality defect known as pale, soft and exudative (**PSE**) meat (Dransfield and Sosnicki, 1999; Owens et al., 2000).

Pale, Soft, Exudative meat was originally identified in pork with flaccid texture and unusually light color. Processed meat products made from either pork or poultry PSE meat are often of inferior quality (Aberle et al., 2001) and lower customer acceptability (Fernandez et al., 2002). This has been attributed to an extensive denaturation of proteins in PSE meat resulting in a loss of protein functionalities including solubility, water-holding capacity, and binding properties. Overall, the PSE

meat defect substantially lowers processing yields and causes significant economic loss to the poultry industry (Owens et al., 2009).

In pigs, it is generally accepted that development of PSE pork associates with a rapid rate of postmortem anaerobic glycolysis, resulting in high carcass temperature and rapid decrease in pH, leading to protein denaturation. The accelerated postmortem glycolysis is associated with abnormal Ca^{2+} homeostasis in muscle cells. A Ca^{2+} leak via sarcoplasmic reticulum (**SR**) calcium release channel proteins (ryanodine receptors, **RYR**) in skeletal muscle cells, results from a single point mutation in *RYR1* that changes the amino acid sequence from arginine at position 615 to cysteine (Mickelson et al., 1988b; Fuji et al., 1991; Otsu et al., 1994).

In contrast, a hypersensitivity of the SR calcium release channels between different turkey lines has been suggested (Wang et al., 1999), but no mutation has yet been observed (Chiang et al., 2004). We hypothesized that some turkeys may be PSE-susceptible because of differences in the abundance of key proteins involved in regulation of intracellular [Ca^{2+}]. Recently, relative mRNA abundance of four major genes involved in Ca^{2+} homeostasis in skeletal muscle cells between normal and PSE turkey breast meat has been determined (Sporer et al., 2012). With the onset of heat stress, the PSE meat showed a significant delay in the up-regulation of *RYR* isoforms, *αRYR* and *βRYR*, and of calsequestrin, the high-capacity, low-affinity Ca^{2+} binding protein located in the lumen of the SR. Transcript abundance of the sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 (***SERCA1***) remained unchanged. This

previous study of Sporer et al. (2012) suggests a complex manifestation of changes in gene expression associated with development of PSE in turkey. It is possible that differential expression of unidentified genes other than SR Ca^{2+} regulators may be revealed in PSE turkey. In addition, comparison of gene expression between normal and PSE turkey meat from birds not subjected to heat stress was not examined in the study of Sporer et al. (2012). This information is important to advance our fundamental comprehension at the transcriptional level regarding the development of PSE turkey meat.

A turkey skeletal muscle long oligonucleotide (**TSKMLO**) microarray was constructed with the initial purpose of screening the skeletal muscle transcriptome for candidate genes critical for growth and development. The platform has been subsequently validated and utilized for studies of domestic turkey muscle biology (Sporer et al., 2011a; Sporer et al., 2011b; Nierobisz et al., 2012). Utilization of the TSKMLO platform enables a simultaneous investigation of the expression of thousands of skeletal muscle genes. The objective of the current study was to investigate differential expression in the turkey skeletal muscle transcriptome between normal and PSE meat using the TSKMLO microarray.

2.2 Materials and methods

Experimental animals and sample collection

Breast meat samples utilized in this study were obtained from the study of Chiang et al. (2008). Briefly, turkeys from the randombred control line 2 (**RBC2**), a

line representative of the commercial turkey of the late 1960s and maintained without selection pressure at The Ohio Agricultural Research and Development Center of The Ohio State University (Wooster, OH), were used in this study (Nestor et al., 1967; Nestor, 1977a; Nestor, 1977b). The birds were raised at the Michigan State University (**MSU**) Poultry farm (Chiang et al., 2008). All methods were approved by the Institutional Animal Care and Use Committee (**IACUC**; AUF#: 06/05-081-00). Turkeys at 22 weeks of age were harvested using standard industry practices in the MSU Meat Laboratory. Breast muscle samples from one side of each bird were collected immediately post-bleed, snap-frozen in liquid nitrogen, and stored at -80°C for later isolation of total RNA. Breast muscle samples from the opposite side were processed under commercial conditions. Breast muscle was classified after 24h as "normal," primarily based on high marinade uptake, and secondarily on low cook loss. Conversely, "PSE" samples were grouped by low marinade uptake and high cook loss (Sporer et al., 2012). Six samples for each extreme of normal (average marinade uptake = 45.3%) and PSE (average marinade uptake = 17.2%) characteristics (n = 6) were used for microarray experiments.

RNA isolation

Total RNA was isolated from breast meat samples using Ambion TRI Reagent Solution (Applied Biosystems, Inc., Foster City, CA), and subsequently purified with the Qiagen RNeasy Mini spin column (Qiagen, Inc., Valencia, CA) according to manufacturer's instructions. Quantity of total RNA was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Integrity of total RNA was confirmed using an Agilent 2100 Bioanalyzer (Santa Clara, CA).

Samples with an RNA Integrity Number (**RIN**) equal to or exceeding 8.0 (RIN = 10 is the best) were used for microarray and quantitative real-time PCR (**qPCR**).

Microarray experimental design

The 6K TSKMLO microarrays were used for transcriptome analysis of normal and PSE meat samples. Details of the array design are available at the National Center for Biotechnology Information's Gene Expression Omnibus (**NCBI GEO**) with the platform accession GPL9788. Gene expression between normal and PSE turkey skeletal muscle samples was directly compared. Six biological replicates were run for each meat quality level (n = 6). Dye swapping was performed to minimize dye bias; i.e., in 3 arrays, the normal samples were labeled with Cy3 fluorescent dye (GE Healthcare, Piscataway, NJ) while PSE samples were labeled with Cy5; for the other 3 arrays, the dye assignments were reversed. A total of six arrays were utilized in this study.

RNA amplification and microarray hybridizations

Amplification of RNA samples, microarray preparation and microarray hybridization were performed as described elsewhere (Sporer et al., 2011a). Briefly, 2µg of total RNA was incubated with T7 Oligo$(dT)_{18}$ as primer and reverse-transcribed into cDNA followed by *in vitro* transcription for amplified RNA (aRNA) using an Amino Allyl MessageAmp^{TM} II aRNA kit (Ambion, Austin, TX) according to the manufacturer's instructions. For dye labeling, 10 µg of aRNA samples were dye-coupled with Cy3 or Cy5 fluorescent dye at room temperature in the dark for 1 h.

Dye-labeled aRNA was purified; then, 5 µg of each sample was fragmented into 60–200 nucleotide fragments using RNA Fragmentation Reagents (Ambion, Inc.) at 70°C for 15 min. The fragmented Cy3-labeled aRNA was equally mixed with its Cy5-labeled counterpart. Hybridization of microarray slides was performed in a GeneTac™ Hybridization Station (Genomic Solutions, Ann Arbor, MI) for 18 h. Arrays were washed with a series of wash solutions and dried by centrifugation. Afterward, the arrays were scanned using a Molecular Devices GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA), and image analysis was performed using Molecular Devices GenePix Pro 6.0 software. Array spot intensities were exported as GenePix Results (**GPR**) files for statistical analysis.

Microarray statistical analysis and gene annotation

Dye intensity bias was normalized using "normexp" background correction method based on Ritchie et al. (2007). Normalized data were described as $\log_2$ fluorescent intensities ratio (Cy5/Cy3) or M-value, and statistically analyzed with a linear model using LIMMA (Smyth, 2005). Specific hybridization was confirmed by monitoring fluorescence intensities of negative control and mismatched oligonucleotides (Sporer et al., 2011a). The microarray data were submitted to the NCBI GEO with GEO accession number GSE36660. The oligos were annotated using NCBI BLASTn (http://www.ncbi.nlm.nih.gov).

Confirmation of expression patterns

Thirteen genes were selected for further analysis by qPCR to confirm the microarray gene expression results. Of 13 genes, 12 genes were chosen based on their large fold changes (**FC**; magnitude of FC indicates ratio of expression of particular gene in PSE relative to normal sample; negative FC indicates decreased expression of the gene in PSE samples or down-regulation; positive FC indicates increased expression in PSE samples or up-regulation) obtained from the microarray study. In addition, profilin (***PFN***) was selected as it showed statistical significance in expression between normal and PSE sample (estimated false discovery rates, **FDR** < 0.1).

Primers (Table 2.1) were designed using Primer Express 3.0 software (Applied Biosystems) and synthesized by Operon Inc. (Huntsville, AL). The confirmation protocol was as described in Sporer et al. (2011a). Briefly, 5 µg of total RNA from the same samples utilized for microarray was reverse transcribed to cDNA using Superscript III (Invitrogen). The cDNA was purified using ethanol precipitation and quantified with a Nanodrop ND-1000 spectrophotometer. Reactions included 10 ng cDNA, 300 nM primer mix, and POWER SYBR Green Master Mix (Applied Biosystems) and were run in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Threshold cycle was analyzed using Sequence Detection Systems Software (**SDS**) version 2.3 (Applied Biosystems). Beta-actin (**ACTB)** was used as an endogenous control gene (Sporer et al., 2012). Relative expression of genes of interest in PSE samples relative to normal samples was

calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Student's t-test was performed to evaluate significant difference ($P < 0.05$) in gene expression between PSE and normal samples.

Pathway analysis

To identify biological functions of the annotated genes, pathway analysis was performed using Ingenuity Pathway Analysis (**IPA**) (Ingenuity® Systems, www.ingenuity.com). Two criteria, FC and FDR, were used to select the genes for pathway analysis. Genes with FC < -1.66 or FC > 1.66 and FDR < 0.35 were uploaded to the IPA. Canonical pathways associated with focus genes were generated from the software.

Table 2.1 Primer information for genes chosen for confirmation of expression using qPCR

Gene Symbol	Primer	Amp Length (bp)	Amp Tm (°C)
ACTB (β-actin)	**F:** GTCCACCTTCCAGCAGATGTG **R:** CAATGGAGGGTCCGGATTC	71	79
ANKRD1	**F:** CGCCGATGCATGATGCT **R:** AATCAAAAGCCGGACCATTTT	56	81
ATP1B4	**F:** TACCCTGGAAACGGCACATT **R:** TGTAGTTGACGTGCGTGAGCTT	70	80
CA3	**F:** CAACCTGATGGTGTGGCTGTT **R:** TCTGGTTTGGGAGTTTTTCCA	62	79
CCDC135	**F:** ATTACCTGGCACCTTTTCTTATTCA **R:** CGGAGGGCCTGCCTTTT	67	79
COL6A1	**F:** TTCCATTGGTGCTCTTGCTATG **R:** TTTGGGATGATGGCGATACC	79	78
CST3	**F:** GTGATCTCCAGAGCTGCGAAT **R:** ACAAAGGTGCATGTGGTATACTTAGC	67	80
IGFN1	**F:** TCCCTGGTGATCTTTAGTGTTTCC **R:** CCTGAATCATTGGTGGCTTCA	71	79
MYH4	**F:** AGCAGGCATTCACCCAACA **R:** GTCATGACGAGCAGACTGCAA	104	82
MYOM1	**F:** TGAGCCAACTCCACAAGACAAA **R:** ATCAAATGCTTGCCCAGAAAGA	100	76
PDK4	**F:** ATGAATGTCTGTAATAGTGCTTGCAA **R:** CATGTCTTCATTGTATGTTCTGCATATAC	90	74
PFN2	**F:** CGGTCTTTCTGCCAGATCACT **R:** CGTCCAAACAGCGGCTTT	65	82
RGS2	**F:** AGGCTCCCAAGGAGATAAACATT **R:** TCCTGGAGGTTCTGTGCTATCA	64	79
TNNT3	**F:** CCCGTGCCTCAGTGATAACTAAA **R:** AGAAGAAAAGCAGCAGCAATAGC	68	78

2.3 Results

Differential gene expression between normal and PSE turkey skeletal muscle

Differential expression of 49 transcripts between PSE and normal turkey skeletal breast muscle was identified (FDR < 0.1). Seventeen transcripts were down-regulated and 32 transcripts were up-regulated (Table 2.2). Among those transcripts, there were two corresponding to oligos annotated as alpha sarcoglycan (***SGCA***), three oligos annotated as myosin heavy chain isoform 2 (***MYH2***), two oligos annotated as nebulin (***NEB***), four oligos annotated as myosin heavy chain isoform 1 (***MYH1***), two oligos annotated as titin (***TTN***) and one unknown oligo (Table 2.3). The top 10 down- and up-regulated genes (based on FC only) are shown in Table 2.4. Down-regulated genes refer to the genes expressed lower in PSE relative to normal samples. Conversely, up-regulated genes are those expressed higher in PSE compared to normal sample.

Table 2.2 Number of differentially expressed transcripts between PSE and normal turkey skeletal muscle at different FDR significance levels

	Number of transcripts		
	Downregulated	**Upregulated**	**Total**
FDR < 0.05	10	19	29
FDR < 0.10	17	32	49
FDR < 0.15	32	43	75
FDR < 0.20	47	57	104
FDR < 0.25	74	71	145
FDR < 0.30	101	97	198
FDR < 0.35	131	113	244

Table 2.3 Differentially expressed transcripts between PSE and normal turkey skeletal muscle (FDR < 0.1)

GenBank Accession#	Gene symbol	Gene name	FC	FDR
DQ993255	*MHC*	Major histocompatibility complex, B locus	-14.6	0.003
NM_001044651	*ATP1B4*	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	-12.0	0.04
NM_204228.1	*MYH2*[1]	Myosin, heavy chain 2, skeletal muscle	-7.1	0.07
XM_419317.3	*RIN2*	Ras and Rab interactor 2	-4.2	0.08
XM_003209725	*CCDC135*	Coiled-coil domain containing 135	-3.6	0.04
NM_001024577	*LINGO1*	Leucine rich repeat and Ig domain containing 1	-2.7	0.03
XM_429975	*STBD1*	Starch binding domain 1	-2.4	0.08
NM_001242311	*ATL2*	Atlastin GTPase 2	-2.3	0.04
XR_118375	*ACSF3*	Acyl-CoA synthetase family member 3	-2.3	0.08
XM_003203019	*USP9X*	Ubiquitin specific peptidase 9, X-linked	-2.1	0.09
NM_001006494	*PSMC6*	Proteasome (prosome, macropain) 26S subunit, ATPase, 6	-2.0	0.01
XM_423397	*SELENBP1*	Selenium binding protein 1	-2.0	0.04
NM_001079760	*PFN2*	Profilin 2	-2.0	0.02

Table 2.3 (cont'd)

GenBank Accession#	Gene symbol	Gene name	FC	FDR
NM_001006260	*EIF2S3*	Eukaryotic translation initiation factor 2, subunit 3 gamma	-1.9	0.08
XM_003207146	*HNRNPA2B1*	Heterogeneous nuclear ribonucleoproteins A2/B1	-1.9	0.02
XM_003209920	*VPS4*	Vacuolar protein sorting-associated protein 4A	-1.8	0.02
XM_003213364	*AQP3*	Aquaporin-3	1.8	0.09
NM_205177	*CTSD*	Cathepsin D	1.9	0.01
XM_003643520	*SGCA*[2]	Alpha sarcoglycan	1.9	0.08
NM_001030899	*THRAP3*	Thyroid hormone receptor associated protein 3	2.0	0.04
XM_003643520	*SGCA*[2]	Alpha sarcoglycan	2.4	0.02
AJ419877		18S rRNA gene	2.4	0.08
XR_118187	*PHKA1*	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform	2.5	0.07
XM_003203043	*RPS6KA3*	Ribosomal protein S6 kinase, 90kDa, polypeptide 3	2.5	0.049
XM_003207155	*TAX1BP1*	Tax1 (human T-cell leukemia virus type I) binding protein 1	2.6	0.08
NM_205096	*HDLBP*	High density lipoprotein binding protein (vigilin)	2.8	0.02
XM_415578	*MYH2*[1]	Myosin, heavy chain 2, skeletal muscle, adult	2.8	0.02
BC067379		DR1-associated protein 1 (negative cofactor 2 alpha)	3.2	0.004
AB024330	*NEB*[3]	Nebulin	3.3	0.02
NM_001013396	*MYH1*[4]	Myosin heavy chain isoform 1, skeletal muscle, adult	3.7	0.004
XM_003204036	*ACTA1*	Actin, alpha skeletal muscle	3.7	0.08
NM_001012945	*DNAJA1*	Heat shock protein 40 kDa homolog, subfamily A, member 1	3.8	0.02
XM_001236426	*HDGF*	Hepatoma-derived growth factor	3.8	0.02

Table 2.3 (cont'd)

GenBank Accession#	Gene symbol	Gene name	FC	FDR
XM_415578	*MYH2*[1]	Myosin, heavy chain 2, skeletal muscle	3.9	0.08
NM_204289	*HSP90B1*	Heat shock protein 90kDa beta (Grp94), member 1	3.9	0.08
XM_420181	*PRPS1*	Phosphoribosyl pyrophosphate synthetase 1	3.9	0.08
NM_001031489	*HBE1*	Hemoglobin subunit beta	4.1	0.02
XR_118264	*TTN*[5]	Titin	4.2	0.08
NM_001013396	*MYH1*[4]	Myosin heavy chain isoform 1, skeletal muscle, adult	4.2	0.01
DQ018757		Clone AY006 28S ribosomal RNA gene, partial sequence.	4.9	0.04
NM_001013396	*MYH1*[4]	Myosin heavy chain isoform 1, skeletal muscle, adult	5.0	0.08
AB024330	*NEB*[3]	Nebulin	5.8	0.04
NM_204959	*MYOM1*	Myomesin 1	7.4	0.02
FM165415		28S rRNA gene, clone GgLSU-1	7.4	0.04
XR_118264	*TTN*[5]	Titin	8.0	0.08
NM_001013396	*MYH1*[4]	Myosin heavy chain isoform 1	8.1	0.01
XM_418319	*CA3*	Carbonic Anhydrase 3	14.6	0.06
EF153719		Mitochondrion, complete genome	18.9	0.04
		Unknown	-2.1	0.09

FC = Fold change

[1] Different oligos on the microarray but were annotated as myosin heavy chain isoform 2.

[2] Different oligos on the microarray but were annotated as sarcoglycan.

[3] Different oligos on the microarray but were annotated as nebulin.

[4] Different oligos on the microarray but were annotated as myosin heavy chain isoform 1.

[5] Different oligos on the microarray but were annotated as titin.

Table 2.4 Top ten down-regulated (A) and top ten up-regulated (B) genes (based on fold change alone) in PSE relative to normal turkey skeletal muscle revealed by the TSKMLO microarray

A. Down-regulated Genes

Symbol	Gene name	FC	FDR
MYH4	Myosin, heavy chain 4, skeletal muscle	-26.2	0.11
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	-25.9	0.33
MHC	Major histocompatibility complex, B locus	-14.6	0.003
MB	Myoglobin	-13.8	0.25
ATP1B4	ATPase, Na+/K+ transporting, beta 4 polypeptide	-12.0	0.04
ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)	-6.8	0.15
RSG2	Regulator of G-protein signaling 2, 24kDa	-4.7	0.41
IGFN1	Immunoglobulin-like and fibronectin type 3 domain containing 1	-4.2	0.14
TNNT3	Troponin T type 3 (skeletal, fast)	-3.8	0.41
CCDC135	Coiled-coil domain containing 135	-3.6	0.04

B. Up-regulated Genes

Symbol	Gene name	FC	FDR
CA3	Carbonic anhydrase III, muscle specific	14.6	0.06
MYH1[1]	Myosin, heavy chain 1, skeletal muscle, adult	8.1	0.01
TTN	Titin	8.0	0.08
MYOM1	Myomesin 1	7.4	0.02
TNNC1	Troponin C type 1 (skeletal muscle, slow)	7.0	0.34
NEB	Nebulin	5.8	0.04
COL6A1	Collagen, type VI, alpha 1	5.7	0.25
CST3	Cystatin C	4.3	0.37
MYH1[1]	Myosin, heavy chain 1, skeletal muscle, adult	4.2	< 0.01
HBB	Hemoglobin, beta	4.1	0.02

FC = Fold change
FDR = False discovery rate
[1] Different oligos on the microarray but were annotated as myosin heavy chain isoform 1

Confirmation of microarray results by qPCR

Confirmation of microarray analysis was performed by conducting qPCR analysis of 13 selected genes (Figure 2.1). The majority of the selected genes showed statistical significance in expression between normal and PSE turkey ($P < 0.05$) with a similar direction as observed in microarray analysis. Five genes including regulator of G-protein signaling 2 (***RGS2***), immunoglobulin-like and fibronectin type 3 domain containing 1 (***IGFN1***), cystatin C (***CST3***), troponin T type 3 (***TNNT3***), and myomesin 1 (***MYOM1***), did not show statistically significant differential expression in the qPCR experiments. It should be noted that, among those five genes, the FDR from the microarray study for *RGS2, CST3* and *TNNT3* exceeded 0.35.

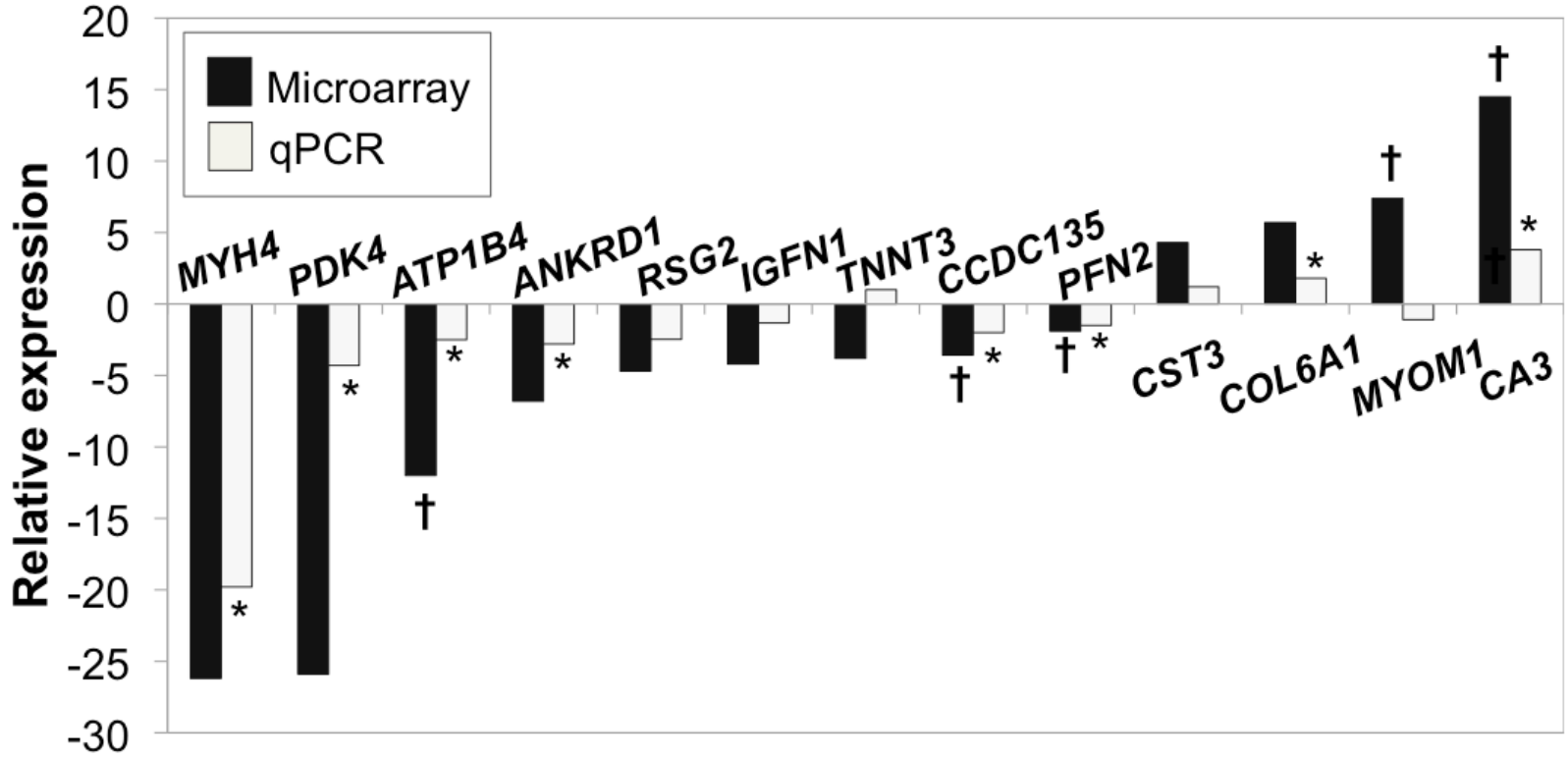


Figure 2.1 Confirmation of gene expression analyzed by microarray using qPCR. Results are presented as relative expression or fold change for gene expression in PSE relative to normal samples. Bars below the origin indicate lower expression (down-regulation) of the gene in PSE samples; bars above the origin indicate higher expression (up-regulation) in PSE samples. Statistical significance indicates change in expression between PSE and normal samples within each technique († FDR < 0.1 for microarray, * P < 0.05 for qPCR).

Functional and pathway analysis using Ingenuity Pathways Analysis (IPA)

In order to perform pathway analysis, two parameters were utilized for gene selection from the microarray results. The primary criterion was FC < -1.66 or FC > 1.66. The second criterion was FDR < 0.35. Using these criteria, expression data from 174 transcripts were uploaded to IPA. According to the IPA knowledge base, 86 genes were recognized and mapped into canonical pathways (well-characterized cellular signaling pathways). Canonical pathways associated with development of PSE turkey are shown in Table 2.5. The calcium signaling pathway (Figure 2.2) was the first pathway suggested by the IPA and supports an association between development of PSE meat and abnormal Ca^{2+} homeostasis. Ras homology family member A (**RhoA**) signaling and actin cytoskeleton signaling (Figure 2.3) were also identified. As suggested by IPA, changes in gene expression regarding development of PSE turkey also altered pathways related to development of muscle and muscle contraction.

Table 2.5 Top canonical pathways associated with development of PSE turkey

Canonical Pathways	Gene symbol	FC	FDR
1. Calcium Signaling	*MYH4*	-26.1	0.11
	ATP2A1	-2.0	0.25
	TNNI2	2.9	0.16
	ACTA1	3.7	0.08
	TNNC1	7.0	0.34
2. RhoA Signaling	*MYLK2*	-2.1	0.28
	PFN2	-2.0	0.02
	IGF1	2.0	0.28
	ACTA1	3.7	0.08
3. Hypoxia Signaling in the Cardiovascular System	*P4HB*	1.8	0.28
	UBE2D2	2.0	0.17
	HSP90B1	3.9	0.08
4. Caveolar-mediated Endocytosis Signaling	*FLNC*	-2.0	0.25
	ITGA7	-1.7	0.25
	ACTA1	3.7	0.08
5. Protein Ubiquitination Pathway	*USP9X*	-2.1	0.09
	PSMC6	-2.0	0.02
	UBE2D2	2.0	0.17
	DNAJA1	3.8	0.02
	HSP90B1	3.9	0.08
6. Nuclear factor-erythroid 2-related factor (NRF2)-mediated Oxidative Stress Response	*DNAJA4*	-2.9	0.23
	AOX1	-1.9	0.25
	DNAJA1	3.8	0.02
	ACTA1	3.7	0.08
7. Integrin Signaling	*MYLK2*	-2.1	0.28
	ITGA7	-1.9	0.25
	CAPN3	2.1	0.18
	ACTA1	3.7	0.08
8. Actin Cytoskeleton Signaling	*MYH4*	-26.2	0.11
	MYLK2	-2.1	0.28
	PFN2	-2.0	0.02
	ACTA1	3.7	0.08

FC = Fold change

FDR = False discovery rate

2.4 Discussion

Development of PSE in poultry poses one of the greatest challenges to the meat processing industry (Anthony, 1998; Petracci and Cavani, 2012; Samuel et al., 2012). The prevalence of PSE turkey meat can be as high as 50% in commercial plants depending on the flock, time of the year and other factors such as transportation (Barbut, 1998; Woelfel et al., 2002). However, even a small amount of PSE turkey entering the processing line can have significant negative effects on quality of the final meat products.

Several investigators have attempted to define PSE meat by using objective measurements to establish a cut-off value that would separate PSE meat from normal (Barbut, 1998; Garcia et al., 2010; Eadmusik et al., 2011). One of the indicators frequently studied is carcass color, as this characteristic can be measured rapidly and is amendable to use on the processing line. However, this indicator is weakly correlated with percent marinade uptake (Chiang et al., 2008) and water holding capacity (Samuel et al., 2012). Thus, use of carcass color for on-line sorting of PSE from normal meat is problematic for the meat industry.

The overall goal of this study is to gain a greater mechanistic understanding of the development of PSE in turkey so that new strategies can be developed to identify PSE-susceptible animals in the breeding population, and thus reduce the number of PSE birds entering the processing line. Turkeys from the RBC2 line, developed without selection pressure, were chosen for this study. Analysis of normal

and PSE breast samples from this line will serve as a basis for comparison with growth-selected turkey lines in future studies.

In previous studies, delayed up-regulation of Ca^{2+} regulatory proteins was observed in PSE turkey samples upon heat stress treatment (Sporer et al., 2012). The results suggested a greater complexity of the development of the PSE meat defect than a single gene mutation. It is possible that additional genes may be involved that have not yet been identified. Unlike the study of individual gene expression, the microarray technique enables simultaneous analysis of thousands of genes with the capability of revealing differential expression of unidentified genes and interaction among genes.

In this study, the 6K TSKMLO microarray was utilized to identify relative transcript abundance between normal and PSE turkey meat samples. Considering the top 10 down- and up-regulated genes, several genes displayed large FC but were not significantly different based on statistical criteria (FDR < 0.1); examples include myosin heavy chain isoform 4 (***MYH4***, FC = -26.2, FDR = 0.11) and pyruvate dehydrogenase kinase, isozyme 4 (***PDK4***, FC = -25.9, FDR = 0.33).

The MicroArray Quality Control (**MAQC**) project suggested a rationale for gene selection (Shi et al., 2008). After completing statistical analysis of numerous platforms, this group found that selection of genes based on a combination FC ranking and a less stringent *P* threshold improves reproducibility and specificity of microarray analyses. The more stringent the *P* cutoff utilized for gene selection, the less reproducible the list of differentially expressed genes (Shi et al., 2008). Based

on this project, a FC cutoff (FC < -1.66 or FC > 1.66) with a less stringent FDR (FDR < 0.35) was used in the present study to identify genes for more confidently performing pathway analysis. Using these expanded criteria, changes in gene expression for several of the selected genes, including *MYH4* and *PDK4,* were confirmed by qPCR (P < 0.05).

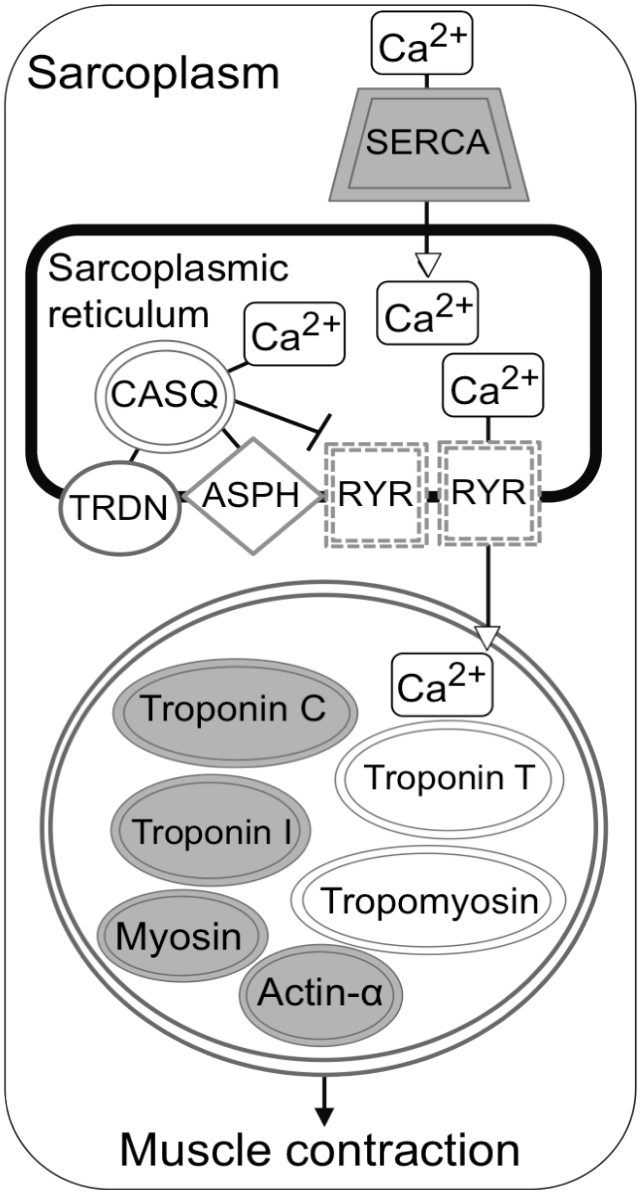
Pathway analysis revealed that several cellular signaling pathways are associated with the development of PSE turkey meat, with numerous genes associated with more than one pathway. For example, *MYH4* was mapped into calcium signaling, actin cytoskeleton signaling, and protein kinase A signaling pathways. This result indicates interactions among molecular pathways associated with development of this meat defect. The potential roles of the calcium signaling, RhoA signaling and actin cytoskeleton signaling pathways in development of PSE meat are discussed below.

Calcium signaling pathway

The calcium signaling pathway (Figure 2.2) was the top pathway highlighted by the IPA. This pathway is of particular interest because several previous studies have implicated abnormal Ca^{2+} homeostasis in the development of PSE meat. In pigs, it is widely accepted that development of PSE pork is associated with a single point mutation in *RYR1,* which results in abnormal Ca^{2+} homeostasis in skeletal muscle of MH-susceptible pigs (Mickelson et al., 1988b; Fuji et al., 1991; Otsu et al., 1994). The rate of Ca^{2+} release from SR in stress-susceptible pigs is about two

times greater than that of normal pigs (Mickelson and Louis, 1996). The high level of sarcoplasmic Ca^{2+} postmortem activates muscle hypermetabolism and accelerates pH decline. With the combination of high acidity and high carcass temperature in the initial postmortem phase, proteins undergo denaturation, causing the PSE meat defect.

Previously, Sporer et al. (2012) found a delay in up-regulation of *αRYR* and *βRYR* expression in PSE turkey compared with normal samples when the birds underwent heat stress. However, in the current study, there was no evidence of differential gene expression of *RYR,* either α-, or β- isoform, between normal and PSE turkey meat when the birds were not undergoing heat stress. This discrepancy may be due to the fact that the fold-change differences observed by qPCR were modest and thus not determined to be significant by the microarray method. These results agree with the study of Oda et al. (2009) who observed an unchanged expression of broiler *αRYR,* although they found decreased expression of *βRYR*. This may be due to the biological differences among species.



Gene Symbol	FC
MYH4	-26.1
ATP2A1	-2.0
TNNI2	2.9
ACTA1	3.7
TNNC1	7.0

Figure 2.2 Schematic diagram of the calcium signaling pathway associated with development of PSE turkey. The pathway, suggested by the IPA, showed direct interaction among differentially expressed genes associated with regulation of Ca^{2+} concentration between normal and PSE turkey skeletal muscle. (FC = fold change, negative FC = down-regulation in PSE samples, positive FC = up-regulation in PSE samples)

Apart from RYR, the intracellular [Ca^{2+}] is also regulated by the SERCA, the SR-Ca^{2+} pump. The SERCA isoform 1 is expressed exclusively in fast-twitch skeletal muscle and is encoded by the ATPase, Ca^{2+}-transporting gene (***ATP2A1***) (Lytton et al., 1992). Mutation of *ATP2A1* results in a reduction of SERCA1 activity and is associated with an exercise-induced impairment of skeletal muscle relaxation and severe cramps in humans (Odermatt et al., 2000) and cattle (Sacchetto et al., 2009). In this study, lower *ATP2A1* expression was found in PSE samples (FC = -2.0). This may imply an overload of intracellular Ca^{2+} causing a severe muscle contraction in the susceptible birds. It should be noted that expression of the gene encoding SERCA1 in PSE turkey remained unchanged with the onset of heat stress (Sporer et al., 2012); however, differential expression of this gene between normal and PSE turkey without heat stress treatment was not determined in the previous study.

Pathway analysis clustered genes encoding myofibrillar proteins within the calcium signaling pathway. Expression of *MYH4* encoding myosin heavy chain (**MHC**) isoform IIb (Tonge et al., 2010) dramatically decreased in PSE samples (FC = -26.1). In contrast, alpha actin (***ACTA1***) was up-regulated (FC = 3.7). Myosin and actin are major contractile proteins in skeletal muscle, which constitute approximately 45% and 20% of myofibrillar proteins, respectively (Aberle et al., 2001). By regulating myofibrillar assembly, changes in expression of genes encoding myosin and actin may affect myofibril accumulation and stability (Wells et al., 1996) and potentially lead to irregular organization of muscle fibers in PSE meat

as previously found in PSE pork (Laville et al., 2005; Obi et al., 2010) and PSE chicken (Wilhelm et al., 2010). Interestingly, while *MYH4* (which encodes fast-twitch glycolytic MHCIIb) was down-regulated, *MYH2* (which encodes fast-twitch oxidative glycolytic MHCIIa) and *MYH1* (intermediate between type IIa and IIb MHCIIx) showed up-regulation. Because turkey breast muscle mainly comprises fast-twitch glycolytic muscle fibers (Rosser et al., 1996), it can be hypothesized that the change in transcript abundance of *MYH* gene family members in the PSE turkey may cause fast-to-slow muscle transformation which is associated with various functional changes at the muscle cell level as well as cytosol-regulating Ca^{2+} dynamics (Kaprielian et al., 1991; Jakubiec-Puka et al., 1999; Pette and Vrbová, 1999; Pette and Staron, 2001; Tonge et al., 2010). However, previous reports on the relative proportion of myosin heavy chain isoforms in PSE meat have not been consistent (Ryu and Kim, 2006; Franck et al., 2007; Golding-Myers et al., 2010).

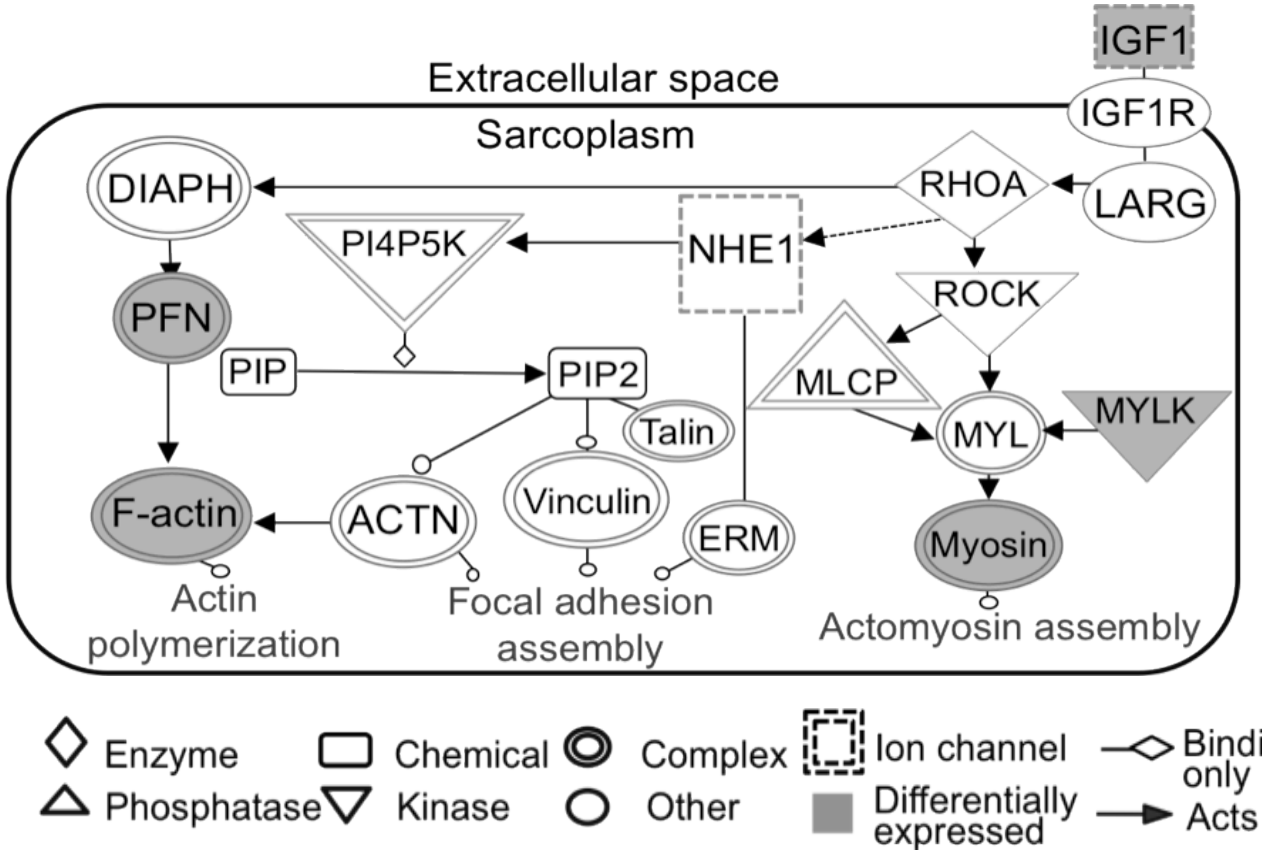
Up-regulation of slow-muscle troponin C (***TNNC1***, FC = 7.0) corresponds to a shift of myosin isoforms, supporting the hypothesis of the fast-to-slow muscle type conversion. Biological properties of slow-muscle troponin C, including Ca^{2+} binding, the Ca^{2+}-bound conformation as well as interaction with troponin I, differ from that of the fast muscle isoform (Sia et al., 1997). In addition, fast-twitch troponin I (***TNNI2***, FC = 2.9) was up-regulated in PSE samples. The differential expression of the regulatory proteins in turkey skeletal muscle may result in change in the ratio of regulatory proteins after protein translation. Together, changes in expression of myofibrillar proteins can directly and indirectly alter interactions among the proteins

and their response to Ca^{2+} flux, with the net effect being altered meat quality.

RhoA signaling and actin cytoskeleton signaling pathway

In this study, an alteration of pathways involved in regulation of arrangement of actin and actomyosin in PSE turkey meat was identified. These pathways include RhoA signaling and actin cytoskeleton signaling (Figure 2.3). RhoA, is a subtype of the Ras superfamily, a low-molecular-weight phosphoprotein family of GTPases (McClung et al., 2004) that links extracellular growth signals or intracellular stimuli to the assembly and organization of the actin cytoskeleton (Schmidt and Hall, 1998).

Insulin-like growth hormone 1 (**IGF1**) was mapped by IPA into the RhoA signaling pathway. This protein, structurally similar to insulin, acts via either autocrine or paracrine mechanisms (McMurtry et al., 1997) and regulates tissue growth and development in various vertebrates (Jones and Clemmons, 1995), including turkeys (Bacon et al., 1993; Richards et al., 2005). Infusion of IGF-1 into chicken has been shown to affect protein synthesis (Conlon and Kita, 2002) and mediate protein degradation (Czerwinski et al., 1998; Tomas et al., 1998). Up-regulation of *IGF-1* in PSE turkey (FC = 2.0) may imply changes in protein turnover, including actin, thus impacting downstream actin cytoskeleton function in the defective meat.



Gene Symbol	FC
MYH4	-26.2
MYLK2	-2.1
PFN2	-2.0
IGF1	2.0
ACTA1	3.7

Figure 2.3 Schematic diagram of RhoA and Actin cytoskeleton signaling pathways associated with development of PSE turkey. The pathway, suggested by IPA, shows interaction among differentially expressed genes associated with polymerization of actin filament and formation of actomyosin complex between normal and PSE turkey skeletal muscle. (FC = fold change, negative FC = down-regulation in PSE samples, positive FC = up-regulation in PSE samples)

The actin cytoskeleton is a polymer of actin monomers assembled together via condensation reaction (Schmidt and Hall, 1998). The primary function of the actin cytoskeleton in skeletal muscle is to tether structural components and maintain overall structural order of those components inside the cell, but in contrast to myofibrillar actin, the cytoskeleton is not directly involved in muscle contraction (Stromer, 1998). The actin cytoskeleton contributes strength and flexibility to accommodate changes in cell shape during muscle contraction. The cytoskeleton is also intimately involved in other cellular functions including cell division and transmembrane signaling (Schmidt and Hall, 1998).

Organization of the actin cytoskeleton is determined by the turnover of actin filaments and actin-binding proteins which are modulated by either internal or by environmental signals (Sheterline and Sparrow, 1994). One of the important actin-binding proteins is profilin encoded by *PFN2*. Profilin catalyzes ATP/ADP exchange of actin monomers and inhibits the hydrolysis of ATP bound to monomeric actin thereby maintaining concentration of readily polymerizing ATP-bound actin (Sohn and Goldschmidt-Clermont, 1994; Dos Remedios et al., 2003). The protein also promotes formation of actin filaments by transporting the monomer to the growing end of the filament (Dos Remedios et al., 2003). In this study, transcript abundance of *PFN2* was lower in PSE samples (FC = -2.0), which may affect the concentration of ATP-bound actin monomers and alter arrangement of actin filaments.

Actin cytoskeleton can be formed along myosin filaments and generate force in the appropriate direction based on polarity of actin filaments. Myosin-based motility of actin cytoskeleton accounts for muscle contraction (when it interacts with muscle

myosin) or cellular morphogenic movement (when it interacts with non-muscle myosin) (Sheterline and Sparrow, 1994). Thus, altered assembly of actomyosin may affect cell motility.

In this study, changes in expression of the gene encoding myosin light chain kinase (**MYLK**) may alter actomyosin assembly. The MYLK is a Ca^{2+}/calmodulin dependent enzyme (Park et al., 2011) that phosphorylates the myosin regulatory light chain (**RLC**). When phosphorylated, the RLC mediates Ca^{2+} sensitivity in myofilaments and promotes movement of myosin cross bridges away from the thick filament surface towards actin filaments (Stull et al., 2011) and affects skeletal muscle twitch potentiation (Manning and Stull, 1982). The MYLK enzyme encoded by the gene *MYLK2* is expressed predominantly in fast-twitch skeletal muscle fibers (Zhi et al., 2005). In the study of Zhi et al. (2005), knockout of the *MYLK2* gene decreased RLC phosphorylation in mouse skeletal muscle. Thus, change in expression of *MYLK2* (FC = -2.1) in PSE meat suggests an alteration of stability of myosin molecules and the actomyosin complex.

Postmortem oxidative metabolism and PDK4

The *PDK4* gene was substantially down-regulated in PSE meat (FC = -25.9). This gene was not mapped by IPA into a specific canonical pathway. However, considering its function, *PDK4* may be one of the key players regarding development of PSE turkey meat. This gene encodes the PDK4 enzyme, one of the regulators of oxidative metabolism in the mitochondria. The enzyme inhibits conversion of pyruvate into acetyl CoA by phosphorylating pyruvate dehydrogenase (Wynn et al.,

2008). The dramatic down-regulation of the *PDK4* gene suggests altered oxidative metabolism in PSE turkey meat. It can be hypothesized that, for normal meat, a small amount of oxygen is still present in the muscle cells at the early stage of postmortem muscle, enabling glucose to undergo oxidative metabolism. When all of the oxygen is consumed, the metabolic pathway switches to anaerobic metabolism to generate ATP for cellular activities. Lactic acid is produced and accumulated, resulting in a pH drop. However, due to drastically lowered expression of the *PDK4* gene, the initial conversion of pyruvate to acetyl CoA and rate of oxygen consumption may be greater in PSE meat. The rate of metabolic switch from aerobic to anaerobic may be faster, resulting in a rapid pH drop that causes protein denaturation in the defective turkey meat. This hypothesis is supported by a lower pH at 15 min postmortem in PSE turkey (Chiang et al., 2008).

Eadmusik et al. (2011) used 20-min postmortem pH to classify turkey breast samples as fast-glycolysing or normal-glycolysing muscle. Samples from the rapid glycolysing group had reduced solubility, which is an indicator of protein denaturation, but the ultimate pH was not significantly different from that of samples with normal rate of glycolysis. Their results support the hypothesis of the current study that although the magnitude of ultimate pH may influence meat quality as previously indicated (Barbut, 1993; Fernandez et al., 2002), a rapid rate of postmortem pH decline, due to decreased expression of *PDK4*, combined with high early-postmortem carcass temperature, may have a greater impact on protein denaturation associated with development of PSE turkey.

Differential expression of *PDK4* has been observed in previous studies on meat quality. Lan et al. (2009) reported reduced mRNA abundance of *PDK4* in muscle from commercial lean-bred Yorkshire pigs compared with a Chinese traditional breed. The authors claimed that the traditional breed has superior meat quality compared to the Yorkshire breed, but they did not directly measure meat quality indices between the two breeds. They suggested that *PDK4* expression is correlated with fiber type; i.e., increased levels of PDK4 would be associated with a higher percentage of slow-twitch fibers. In this study, up-regulation of several genes in PSE turkey has been observed that would suggest a shift in expression from fast-twitch muscle proteins to slow-twitch muscle proteins (Tables 3.3 and 3.4). Slow twitch muscle generally has a greater degree of oxidative metabolism (Donoghue et al., 2007). The results are further consistent with this transition in that the mitochondrial genome was up-regulated in the defective meat (Table 2.3). An increased expression of PDK4 in association with increased expression of oxidative-metabolism genes has been expected to be found. However, the fact that PDK4 was down-regulated in PSE muscle suggests that in this subset of birds, there is impaired regulation of expression of this gene.

In a study on differential expression in chickens differing in glycogen content, Sibut et al. (2011) observed down-regulation of *PDK4* in high-glycogen chicken skeletal muscle compared with low-glycogen samples. They did not find differences in direct measures of meat quality such as drip loss, but the high-glycogen meat showed lower ultimate pH and lighter color (Sibut et al., 2011), determinants often been used as PSE indicators in previous studies (Oda et al., 2009; Ziober et al.,

2010). However, although it is of interest that differential *PDK4* expression was observed in the study of Sibut et al. their results are not directly comparable to the current study. They classified chicken muscle samples based on glycogen content to determine the molecular mechanisms involved in variation of meat quality. In this study, however, samples were first classified as PSE or normal and then analyzed for differential gene expression.

2.5 Conclusion

In conclusion, this study is the first evidence of global differential gene expression between normal and PSE turkey. Pathway analysis shows several molecular signaling pathways associated with development of the turkey meat defect including the calcium signaling pathway which supports an abnormality of Ca^{2+} homeostasis in the susceptible animals. The results also suggest skeletal muscle fast-to-slow isoform switch, which may reflect different molecular properties and disorganization of muscle fibers in PSE turkey. Altered stability of non-muscle actin polymer and actomyosin assembly in PSE turkey is implicated due to differential expression of genes in the RhoA signaling and actin cytoskeleton signaling pathway. Clearly, development of this turkey meat defect is complex and associated with interactions of more than one pathway. Here, evidence was found of dramatically lower expression of the *PDK4* gene in PSE turkey meat, which may alter oxidative metabolism and be associated with an unusually high rate of postmortem metabolism in PSE turkey. Further investigation at the protein level and activity of this enzyme is needed to better understand its role in turkey PSE meat.

CHAPTER 3

DEEP TRANSCRIPTOME SEQUENCING REVEALS DIFFERENCES IN GLOBAL GENE EXPRESSION BETWEEN NORMAL AND PALE, SOFT AND EXUDATIVE TURKEY MEAT

ABSTRACT

An association between development of pale, soft and exudative (PSE) turkey and changes of gene expression has been shown; however, understanding of altered molecular mechanisms associated with the differentially expressed genes remains unclear. The objective of this study was to utilize deep transcriptome RNA sequence analysis (RNA-Seq) to identify differentially expressed genes and the associated molecular mechanisms between normal and PSE turkey breasts. Following collection of turkey breasts (n = 43), meat quality characteristics were determined and samples were classified as normal or PSE based on marinade uptake (high = normal; low = PSE). Total RNA from breast muscle samples with the highest (n=4) and lowest (n=4) marinade uptake were isolated and sequenced using the Illumina GA_{IIX} platform. Of 21,340 gene loci discovered by RNA-Seq, 8480 loci completely matched the turkey reference genome, and 494 genes were differentially expressed (false discovery rate, FDR<0.05). Changes in gene expression were confirmed using quantitative real-time PCR. Pathway analysis of differentially expressed genes suggested abnormalities of calcium homeostasis and signaling pathways regulating actin cytoskeleton structure as well as carbohydrate metabolism and energy production in PSE samples. A dramatically decreased expression of pyruvate dehydrogenase kinase, isozyme 4 may alter early-stage postmortem glucose oxidative metabolism and result in development of PSE turkey meat.

3.1 Background

Over the past 60 years, pale, soft and exudative (**PSE**) meat has been one of the greatest challenges to the poultry meat processing industry. The incidence of PSE turkey has increased in concert with intensive selection of turkeys for rapid growth rate and large breast size (Dransfield and Sosnicki, 1999; Owens et al., 2000; Samuel et al., 2012). Depending on environmental conditions, the incidence can range from < 1% to as high as 50% in commercial processing plants (Woelfel et al., 2002).

The term PSE has been used to describe characteristics of defective meat with unusually light color and inferior water binding properties (Aberle et al., 2001). Myofibrillar proteins of PSE meat undergo irreversible denaturation and lose their most critical functionalities, including protein solubility, extractability and water holding capacity (Pietrzak et al., 1997; Warner et al., 1997). The loss of protein functionality is most readily manifested in processed meat products by excessive purge and structural defects. Therefore, the PSE problem results in substantially reduced processing yields and economic losses in the poultry industry (Owens et al., 2009).

It is widely accepted that the extensive protein denaturation of PSE meat is a consequence of muscle hypermetabolism during early-stage postmortem conversion of muscle to meat. As a result of aberrantly rapid glycolysis, lactic acid is produced which accumulates inside the muscle cell at an accelerated rate, resulting in fast pH

decline. The combination of high carcass temperature and high acidity during the early-stage postmortem period causes protein denaturation (Barbut et al., 2008).

In PSE pork, accelerated postmortem metabolism is associated with an abnormality of the sarcoplasmic reticulum (**SR**) Ca^{2+} release channel protein, ryanodine receptor 1 (**RYR1**) (Mickelson et al., 1988b). Later, Otsu et al. (1994) showed that a single point mutation of *RYR1* is responsible for a leak of the SR Ca^{2+} release proteins.

In turkeys, Chiang et al. (2007) identified alternatively spliced products of *αRYR* (homologous to mammalian *RYR* isoform 1) and single-nucleotide polymorphisms of the *βRYR* isoform (homologous to mammalian RYR isoform 3), suggesting that there may be differences in regulation of intracellular Ca^{2+}. A delay in up-regulation of *αRYR, βRYR* and calsequestrin was also found in PSE turkey meat from birds subjected to heat stress (Sporer et al., 2012).

Recently, global gene expression in PSE and normal turkey meat was profiled using the 6K turkey skeletal muscle long oligonucleotide (**TSKMLO**) microarray (Malila et al., 2013). The results suggested an association between differential expression of genes involved in Ca^{2+} homeostasis with development of PSE turkey. Moreover, microarray analysis revealed a complex manifestation of several affected cellular pathways in addition to calcium signaling that were associated with PSE development in turkey. However, the microarray approach was limited by the fact

that some genes and alternatively spliced products, which are not included on the array, may significantly contribute to the development of turkey PSE.

Deep transcriptome mRNA sequence analysis (**RNA-Seq**) is an emerging technology for transcriptome profiling and offers advantages over microarrays. Unlike hybridization-based microarray, RNA-Seq can quantify the actual gene expression (Wang et al., 2009) and identify transcripts beyond those represented on the microarray (Roh et al., 2010). Thus, RNA-Seq can reveal novel transcripts as well as novel isoforms produced by alternative splicing (Baginsky et al., 2010; Tranpnell et al., 2010). The hybridization-based microarray technique is also limited by dynamic signal range resulting from saturation of the fluorescence signal (Roberts et al., 2011). Additionally, results have shown that RNA-Seq data are more reproducible with smaller technical variation compared to microarray hybridization data (Marioni et al., 2008).

The objective of this study was to utilize RNA-Seq to investigate global gene expression in normal and PSE turkey breast meat. Because RNA-Seq and microarray platforms offer distinct comparative advantages and disadvantages, this RNA-Seq study was designed to augment and complement the results obtained from the microarray study. The present study used RNA samples previously used for the microarray analysis from turkeys of the Randombred Control Line 2 (RBC2), representative of the late 1960s commercial turkeys (Nestor et al., 1967; Nestor, 1977a; Nestor, 1977b) and maintained without selection pressure at The Ohio Agricultural Research and Development Center of The Ohio State University

(Wooster, OH). Analysis of normal and PSE breast samples from the RBC2 line will serve as a basis for future comparisons with modern growth-selected turkey lines.

3.2 Materials and methods

Sample information

Turkey breast muscle samples used in this study comprised a subset of the control sample group collected from RBC2 turkeys in the study of Chiang et al. (2008). Briefly, the birds were slaughtered at 22wk of age using standard industry practices at the Michigan State University Meat Laboratory. Breast muscle was collected, snap frozen and stored at -80°C until utilized for RNA isolation or microsomal membrane protein preparation. Meat quality indices were also determined (Chiang et al., 2008). The samples were classified as normal or PSE based primarily on marinade uptake and secondarily on cook loss as previously described (Malila et al., 2013; Sporer et al., 2012). In brief, normal samples were classified based on high marinade uptake and on low cook loss. Conversely, PSE samples were characterized by low marinade uptake and high cook loss. Four samples with the highest marinade uptake (normal) and four samples with the lowest marinade uptake (PSE) were used (a subset of the samples evaluated by microarray; Malila et al., 2013). Meat quality indices of the samples utilized in this study are shown in Table 3.1.

Total RNA samples were the same materials isolated from each muscle sample, and used for the microarray study (Malila et al., 2013). The RNA isolation procedure was detailed in Malila et al. (2013). Sample quality was assessed using

an Agilent Bioanalyzer and all samples had an RNA Integrity Number (**RIN**) above 8.0 (maximum = 10).

Table 3.1 Meat quality indices of the meat samples utilized in the RNA-Seq study

Meat quality index[1]	Normal	PSE	Significance level[2]
pH (15 min postmortem)	5.77 ± 0.35	5.59 ± 0.19	NS
% Marinade uptake	50.83 ± 9.64	15.19 ± 1.89	***
% Drip loss	0.46 ± 0.30	0.86 ± 0.35	NS
% Cook loss	27.70 ± 2.18	31.16 ± 0.42	*

[1]Meat quality was determined in the study of Chiang et al. (2008). The results shown here are specific for the meat samples defined as normal (n=4) or PSE (n=4) that were utilized in this study.

[2] NS = non-significant ($p>0.05$), *$P<0.05$, **$P<0.01$, ***$P<0.001$

RNA transcriptome sequencing analysis

RNA deep sequence analysis was performed at the Michigan State University Research Technology Support Facility (**RTSF**). Eight RNA libraries (meat quality levels = 2, biological replicates = 4) were constructed from 10 µg of total RNA per sample using the Illumina mRNA Sequencing Sample Preparation Kit (Illumina, Inc., San Diego, CA) according to the maunfacturer's protocol. Briefly, mRNA was purified using oligo-dT beads (Illumina, Inc.) and subsequently fragmented. First strand cDNA was synthesized with random hexamer priming followed by second strand synthesis by random hexamer priming. Ends of the synthesized cDNA were repaired, A-tailed and bar-coded by adapter ligation. The cDNA templates were purified and enriched using PCR. All eight libraries were pooled and sequenced across 4 lanes. Paired-end reads were generated using the Illumina Genome

Analyzer$_{IIx}$ (Illumina, Inc) with 2x55 bp. The resulting mRNA-Seq sequencing reads were aligned to the domestic turkey reference genome (Turkey_2.01, UMD, Ensemble release 61; Dalloul et al., 2010) using TopHat (Trapnell et al., 2009). Alignments produced from each library were used as input to Cufflinks (Trapnell et al., 2010), which assigned reads to isoforms and generated a set of predicted transcripts based on assembly of overlapping reads, which were subsequently transferred to Cuffcompare to be combined into a single, non-redundant set of transcript models. The resulting alignments were compared to an annotation file and the number of fragments uniquely aligned to each gene in the annotation were reported using HTseq-count (http://www-huber.embl.de/users/anders/HTSeq/). Read counts per locus generated by HTseq-count were used as input for Bioconductor R package, DESeq (Anders and Huber, 2010) which subsequently calculated sample variance from biological replicates and tested for differential expression following the standard method outlined in the DESeq documentation. Differential expression was reported as fold change (**FC**) indicating ratio of expression of the gene in PSE samples relative to normal samples. Negative FC denotes down-regulation while positive FC suggests up-regulation. RNA-Seq data have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (**NCBI GEO**) with GEO accession number GSE37176.

Confirmation of expression patterns

Fourteen genes, differentially expressed (false discovery rate; **FDR**<0.05) between normal and PSE samples, were selected for expression confirmation using

quantitative real-time PCR (**qPCR**). Of 14 genes, nine genes were representatives of genes with large fold changes (|FC| > 3.5). The other five were randomly chosen from the group of differentially expressed genes with moderate to low fold change (1.5 < |FC| < 3.0).

Primers (Table 3.2) were designed using Primer Express 3.0 software (Applied Biosystems). Primer specificity was confirmed by subjecting the primer sequence to an NCBI BLAST search. Only primers that specifically matched their respective genes were submitted for primer synthesis by Operon Inc. (Huntsville, AL). Additional specificity was verified by dissociation curves with a single peak at melting temperature and no peak in no-template controls. The qPCR protocol was previously described in Malila et al. (2013).

Functional and pathway analysis

Pathway analysis was performed to identify biological functions of differentially expressed genes between PSE and normal samples. Genes with FDR<0.05 were uploaded into Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com, Ingenuity Systems, Redwood City, CA). Of 494 differentially expressed genes, 402 were recognized by IPA and included in the analysis. Lists of functional categories, canonical pathways, and networks associated with differentially expressed genes were generated.

Table 3.2 Primer information for genes chosen for confirmation of expression identified in the RNA-Seq analysis using qPCR

Gene ID	Primer	Length (bp)	Tm (°C)
ATP1B4	**F:** TACCCTGGAAACGGCACATT **R:** TGTAGTTGACGTGCGTGAGCTT	70	80
ACTB	**F:** GTCCACCTTCCAGCAGATGTG **R:** CAATGGAGGGTCCGGATTC	71	79
CCDC135	**F:** ATTACCTGGCACCTTTTCTTATTCA **R:** CGGAGGGCCTGCCTTTT	67	79
COL6A1	**F:** TTCCATTGGTGCTCTTGCTATG **R:** TTTGGGATGATGGCGATACC	79	78
DCN	**F:** CCCATCGGATGAATATGTGAAA **R:** GCAATGTTCAACTAGGCCTTACTG	96	74
FMOD	**F:** GGCTCTCTACCAACACTTTCAACA **R:** GGTTCCCTTGAAGGTAGAGGTTCT	120	83
FSCN1	**F:** CCGCTCCTCCTATGATGTCTTC **R:** CCCAGTGGTATCCTTGATGTTGT	70	79
FSTL1	**F:** TGTCCTCCAGTGCTGATCTCAA **R:** ATGTAAATATCTGTGACTGGGCAACT	68	78
NOV	**F:** AATGATGGGCGGTGCTGTA **R:** GACAGCGGAACTCAACTTGAATG	61	80
PDE10A	**F:** GGGAGAAAGTAACGAGAGGTGAAG R: GAGGTTCCAGGAGCAATGGA	70	79
PDK4	**F:** ATGAATGTCTGTAATAGTGCTTGCAA **R:**CATGTCTTCATTGTATGTTCTGCATATAC	90	74
PFKFB3	**F:** CCAACTAAAGAGAACAATCCAAACAG **R:** GGGCCTTCCACTGCTCATAG	65	82
POSTN	**F:** CAGGGAGCTGGAACTGAGTACA **R:** TACAGGTGCTTCTTCCAAATGGA	105	77
RGS2	**F:** AGGCTCCCAAGGAGATAAACATT **R:** TCCTGGAGGTTCTGTGCTATCA	64	79
TAGLN	**F:** CCCTCACCGATTGGTATTTATTG **R:** TTGTTGGTCCCCATCTGTAAGC	78	82

Microsomal membrane preparation

Meat samples utilized for microsomal membrane preparation were from the same RBC2 turkey breast muscles previously identified as normal (n = 4) and PSE (n = 4) and used in RNA-Seq analysis. For the membrane preparation, 6 g breast muscle, excised immediately at slaughter and frozen at 80°C, was incubated in 30 mL of Buffer I (0.1 M NaCl, 5 mM Tris-maleate (pH 7.0), 30 mM β-mercaptoethanol) supplemented with protease inhibitor cocktail (0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 µg/mL aprotinin, 1mM benzamidine, 1 µg/mL leupeptin, 1 µg/mL pepstatin A) for 15 min and homogenized using a Waring blender (15 sec on followed by 15 sec rest, 8 cycles). All steps of microsomal membrane preparation were carried out at 4°C. The homogenate was centrifuged at 3,300xg for 30 min and the supernatant was filtered through glass wool and centrifuged at 16,000xg for 30 min. The resulting pellet was re-suspended in 30 mL of Buffer II (0.6 M KCl, 5 mM Tris-maleate (pH 7.0), 30 mM β-mercaptoethanol) supplemented with the protease inhibitor cocktail described above, and gently homogenized using a Dounce homogenizer. The homogenate was centrifuged at 130,000xg for 40 min and the pellet collected and re-suspended in 0.5 mL 10% (w/v) sucrose. Concentration of crude microsomal protein was determined using the Pierce Bicinchoninic acid (**BCA**) Protein Assay kit (Thermo Scientific, Rockford, Illinois) in accordance with manufacturer's recommendation.

Protein electrophoresis

Crude microsomal protein was separated by electrophoresis. Briefly, 40 µg crude microsomal protein was mixed with 3.4 µL of 6X Laemmli sample buffer (Bio-Rad, Hercules, CA). The protein solution volume was adjusted to 20 µL with water and heated at 90°C for 10 min. Samples were separated using a 12% Tris-glycine Criterion gel with dimensions of 133 x 87 x 1 mm (Bio-Rad) at constant 200 V for 1 hr at 4°C. The running buffer was Tris/glycine/sodium dodecyl sulfate (**SDS**) containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3. Pre-stained low-range molecular weight protein standards (Bio-Rad) were used as protein markers.

Immunoblot

After electrophoresis, the gel was equilibrated in 100 mL transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 10 min at room temperature. Proteins were transferred to a methanol-prewetted Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA) using Criterion blotting cell (Bio-Rad) at constant 30 V for 4 h at 4°C. After blotting, the membrane was briefly rinsed with Tris-saline-tween buffer (TBST; 20 mM Tris, 140 mM NaCl 0.1% tween 20, pH 7.4) for 5 min at room temperature, and pre-incubated with 20 mL blocking buffer (LI-COR, Lincoln, NE) for 1 h at room temperature. The membrane was subsequently incubated overnight at 4°C with a mixture of a polyclonal rabbit anti-PDK4 (101-114) antibody (Sigma-Aldrich, St. Louis, MO) mixed with a monoclonal mouse anti-β-actin antibody (Sigma-Aldrich) diluted (1:500 and 1:10000,

respectively) in the LI-COR blocking buffer. The blot was washed using TBST (5 min, room temperature, 4 times) and then incubated with a mixture of secondary antibodies (1:3000 anti-rabbit IgG IRDye 700DX® conjugated antibody, 1:3000 anti-mouse IgG IRDye800® conjugated antibody, Rockland, Gilbertsville, PA) diluted in blocking buffer in the dark for 1 h at room temperature. The membrane was scanned using the Odyssey® Infrared Imaging System (LI-COR). Images were acquired using Odyssey 3.0 analytical software (LI-COR).

For analysis of the immunoblot images, densitometry was performed using ImageJ 1.46r (Rasband, 2012). A rectangular box was manually placed around the band of interest in the first lane. Features with identical size were used to measure the other bands for all lanes. The band intensity of PDK4 was normalized with the same-lane intensity value of β-actin, and the ratios were defined as relative protein abundance. Previous studies in our laboratory have verified β-actin as a valid endogenous control for PSE and normal turkey breast muscle (Unpublished data). The assay was conducted in triplicate; thus, there were three ratios, obtained from different blots, per biological replicate. An average of the three ratios was used as protein abundance of each bird in statistical analysis. Statistical difference in expression of PDK4 between normal and PSE turkey was determined using Student's t test.

3.3 Results

Differential gene expression between normal and PSE turkey skeletal muscle revealed by RNA-Seq

In the current RNA-Seq analysis of turkey breast meat, Cuffcompare assembled mRNA sequence reads into 21,340 transcript loci and assigned Cufflinks class code to each locus (Table 3.3). The class code guided how the Cuffcompare-generated locus matched to the reference gene. The top three class codes were assigned to loci that completely matched with the intron chain (1st class code, "="), or were contained in the reference gene (2nd class code, "c"), or where at least one splice junction of the locus was shared with a reference transcript (3rd class code, "j"). Of 21,340 loci, 8,480 loci were assigned to the first three classes, suggesting a close match of the loci with the reference gene. As a result, this group was subsequently utilized in identification of differential gene expression (Table 3.4).

Table 3.3 Number of loci classified within different Cufflink class code

Cufflink class code	Code priority	Code definition	Number of loci
=	1	locus completely matched with intron chain	3346 (16%)
c	2	locus contained in reference gene	3225 (15%)
j	3	locus is potentially novel isoform and at least on splice junction is shared with a reference transcript	1909 (9%)
e	4	a possible pre-mRNA fragment	216 (1%)
i	5	a transfrag falling within an intron region	2139 (10%)
o	6	generic overlap with reference	357 (2%)
p	7	possible polymerase run-on fragment	1637 (8%)
x	8	exonic overlap with opposite strand of the reference	2745 (13%)
s	9	intronic overlap with opposite strand of the reference likely due to mapping error	451 (2%)
.	10	loci with multiple classifications	5315 (25%)
		Total	**21,340**

Table 3.4 Number of differentially expressed transcripts at different significance level

Significance level	Number of differentially expressed transcripts
FDR < 0.01	326 (3.84%)
0.01 ≤ FDR < 0.05	168 (1.98%)
0.05 ≤ FDR < 0.1	133 (1.57%)
0.1 ≤ FDR < 0.2	210 (2.48%)
0.2 ≤ FDR < 0.3	198 (2.33%)
0.3 ≤ FDR < 0.4	229 (2.70%)
0.4 ≤ FDR < 0.5	397 (4.68%)
FDR ≥ 0.5	6,820 (80%)
Total	**8,481**

Differential expression of 494 loci between normal and PSE turkey skeletal muscle was revealed (FDR<0.05), with 182 down-regulated loci and 312 up-regulated loci (Appendix B). Approximately 80% of the down-regulated loci fall within FC range between -1.5 to -3.0, and the remaining 20% showed FC < -3.0. About 86% of up-regulated loci showed FC within the range of 1.5 to 3.0 with the remaining 14% of up-regulated loci showing FC > 3.0 (Figure 3.1). Among differentially expressed loci, 91 are unknown or uncharacterized proteins. For the identified loci, pyruvate dehydrogenase kinase isoform 4 (***PDK4***) showed the greatest down-regulation (FC = -14.1) in PSE turkey while nephroblastoma overexpressed gene (***NOV***) was the most up-regulated gene (FC = 37.7) in PSE turkey meat. Biological functions and location of the changed genes showing the greatest differential expression are shown in Figure 3.2.

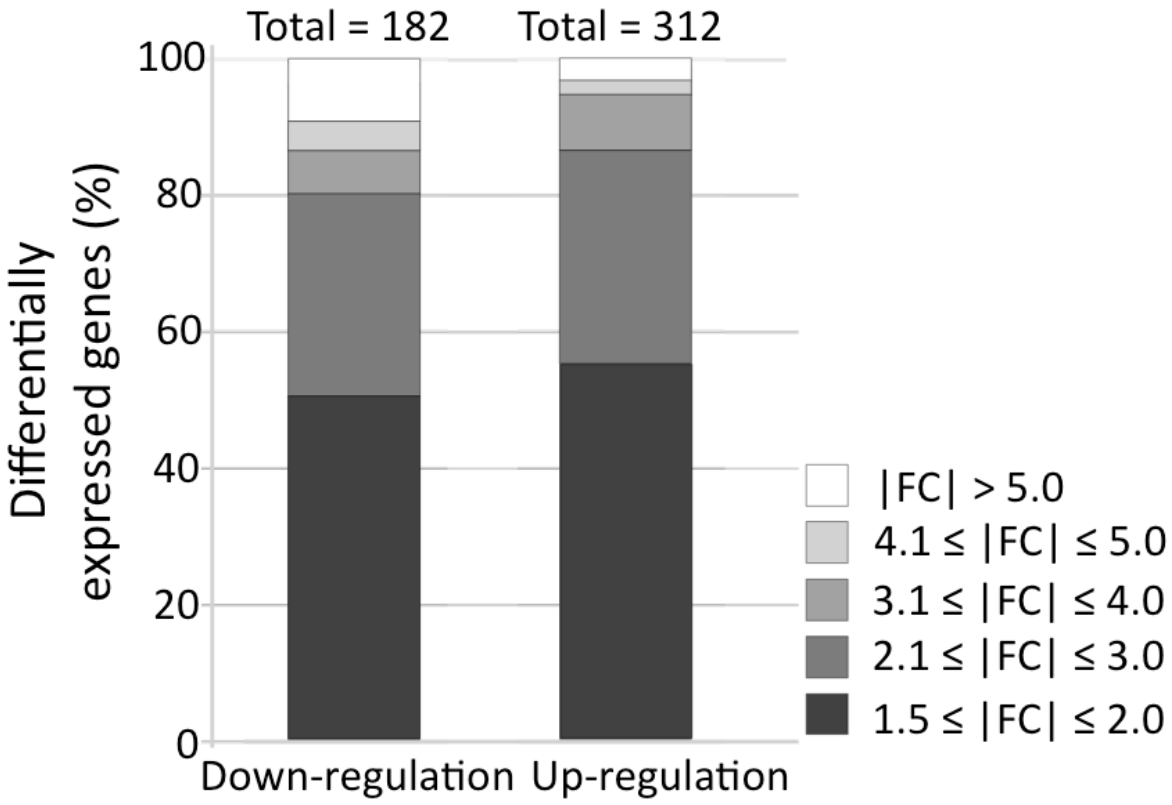


Figure 3.1. Distribution (%) of differentially expressed transcripts (FDR<0.05) at different fold change (FC) ranges. Percentage was calculated separately within down-regulated (total = 182) and up-regulated (total = 312) transcripts. Different shades on bar graphs represent ranges of absolute value of FC (|FC|).

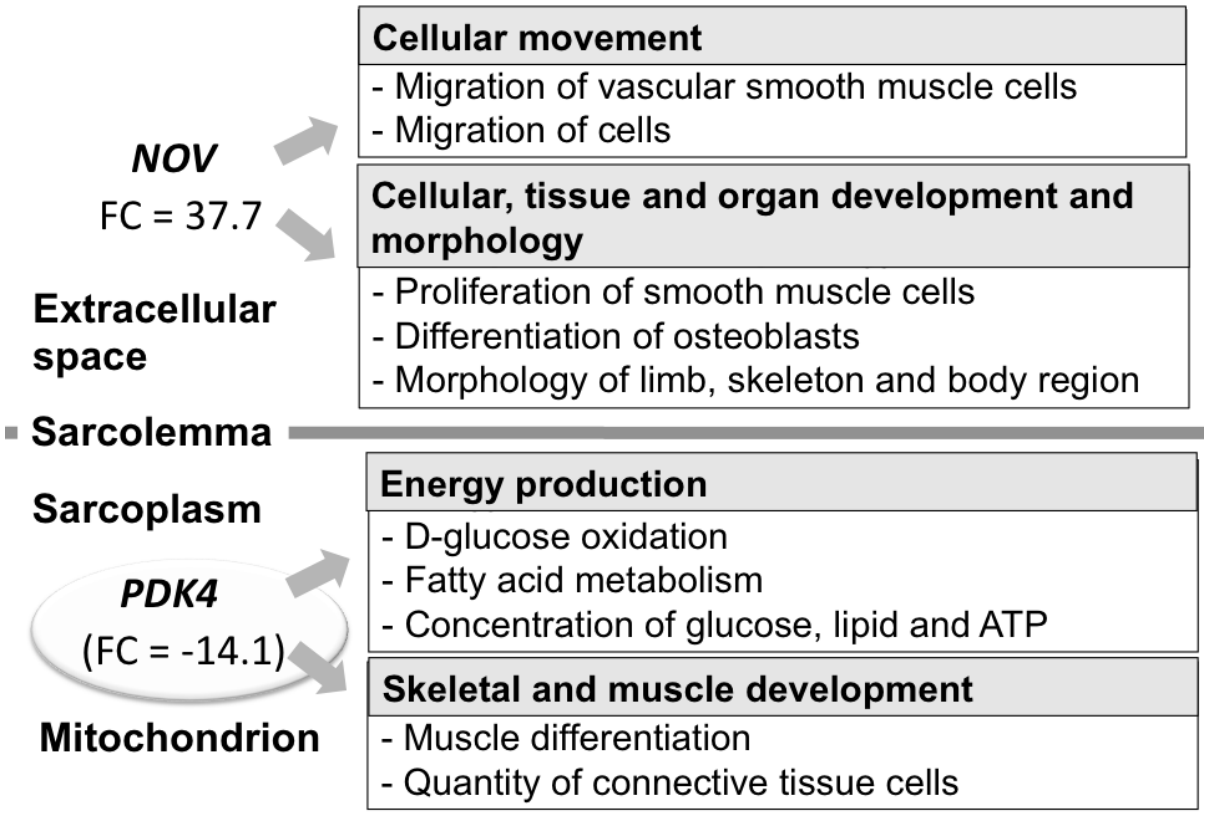


Figure 3.2 Schematic diagram showing localization and associated functions of the most up-regulated and down-regulated genes identified by RNA-Seq. The most up-regulated gene is nephroblastoma overexpressed gene (*NOV*). The most down-regulated gene is pyruvate dehydrogenase kinase isozyme 4 (*PDK4*). FC, fold change or gene expression in PSE relative to normal samples. Negative FC indicates down regulation of gene in PSE samples; positive indicates up-regulation in PSE samples.

Confirmation of gene expression differences by qPCR technique

Differential gene expression, observed by RNA-Seq, was confirmed by qPCR analysis for fourteen genes (Figure 3.3). Overall, the fold changes of most genes analyzed by qPCR were similar to that observed by RNA-Seq at the same significance level ($\alpha = 0.05$). Only fascin (***FSCN***), follistatin-related protein 1 (***FSTL1***), fibromodulin (***FMOD***) and *NOV* showed expression differences between PSE and normal meat (FDR < 0.05) by RNA-Seq but were not significantly different by qPCR analysis. This discrepancy could have resulted from biological variation. Additionally, because of the principles underlying each technique, it is possible that RNA-Seq and qPCR were actually reporting expression of different isoforms but those isoforms were still annotated as the same gene.

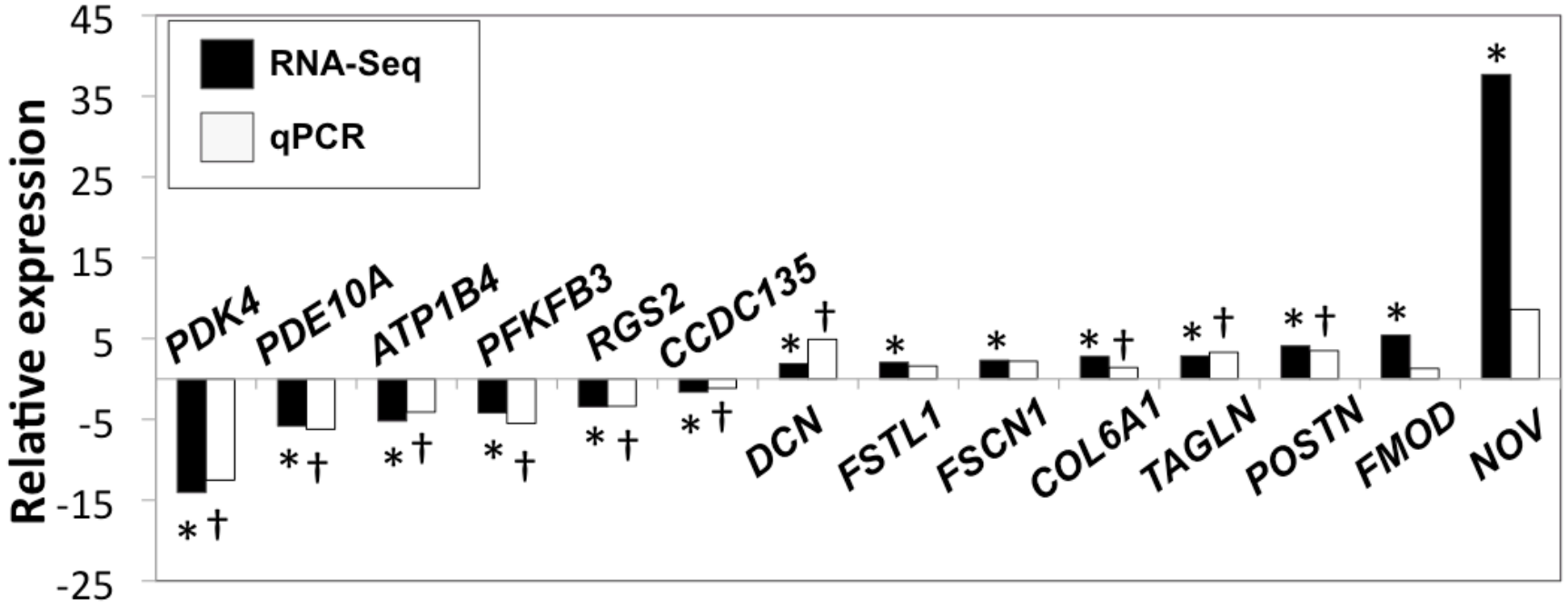


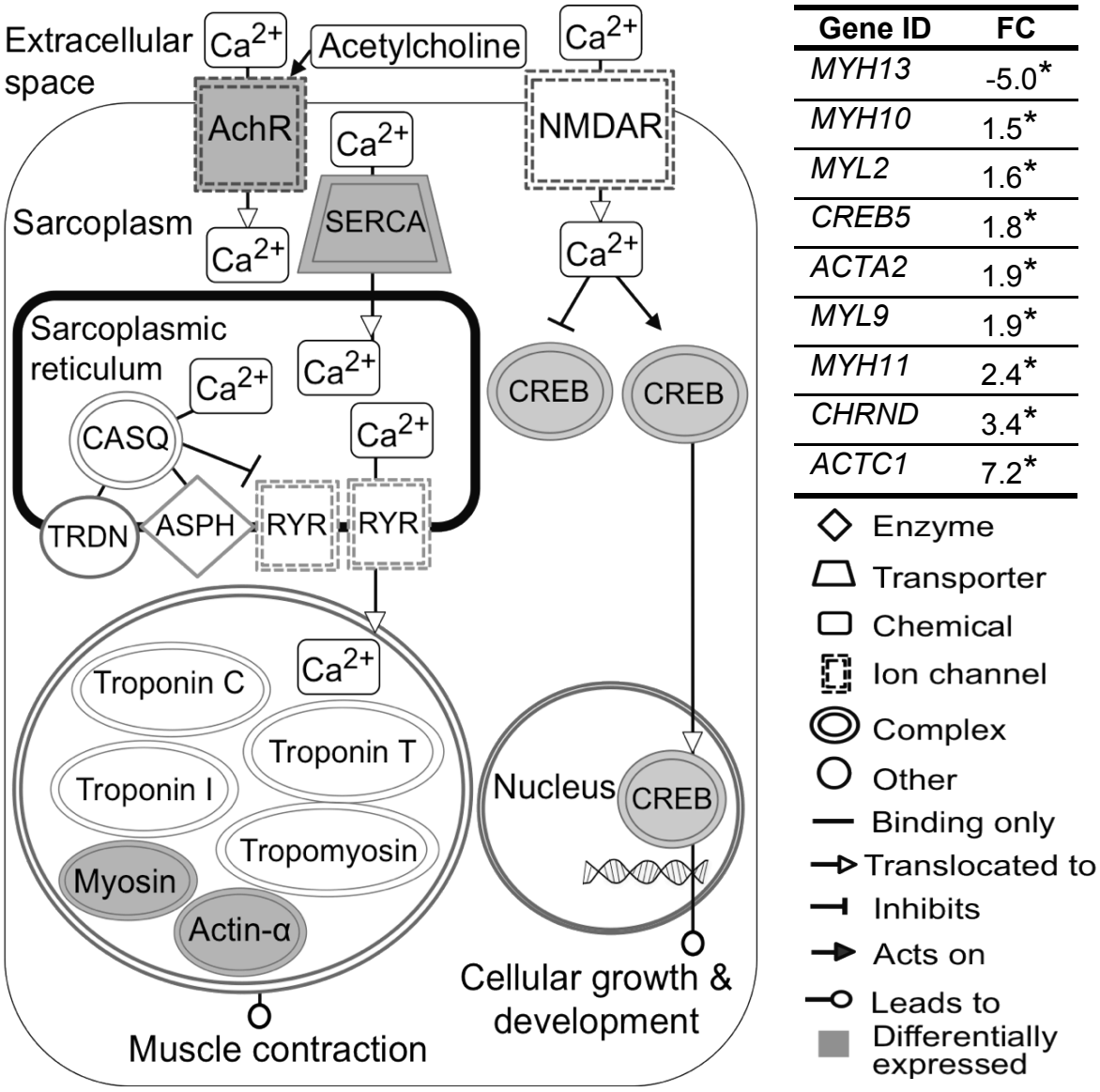
Figure 3.3 Confirmation of gene expression analyzed by RNA-Seq using qPCR. Results are presented as relative expression or fold change for gene expression in PSE relative to normal samples. Bars below the origin indicate lower expression (down-regulation) of the gene in PSE samples; bars above the origin indicate higher expression (up-regulation) in PSE samples. Statistical significance indicates change in expression between PSE and normal samples within each technique (*FDR < 0.05 for RNA-Seq, † P < 0.05 for qPCR).

Functional and pathway analysis

For pathway analysis, 402 genes identified by RNA-Seq and recognized by IPA, were grouped on the basis of biological and functional relationships into canonical pathways, functional networks and cellular and molecular functions.

The current RNA-Seq study confirmed abnormality of calcium signaling pathways (Figure 3.4) associated with development of PSE turkey. Actin cytoskeleton and cell motility-related canonical pathways exhibited a greater degree of complexity of interactions compared to that reported in the microarray study (Malila et al., 2013). The interactions comprise actin cytoskeleton signaling and Ras homology family member A (**RhoA**) signaling, two pathways originally identified in the microarray analysis (Malila et al., 2013), which now include integrin, integrin-linked kinase (**ILK**), focal adhesion kinase (**FAK**), tight junction, epithelial adherens junction and remodeling of epithelial adherens junction signaling pathways (Figure 3.5).

The top three altered biological functions associated with differential gene expression in PSE turkey were cellular movement, cellular assembly and organization, and cellular function and maintenance. These three functional activities potentially increased in the defective meat (Table 3.5), as predicted by IPA based on overall directional changes in expression of genes associated with particular function. Ingenuity Pathway Analysis also predicted likelihood of increased metabolism of carbohydrate and fatty acids in PSE samples.



Gene ID	FC
MYH13	-5.0*
MYH10	1.5*
MYL2	1.6*
CREB5	1.8*
ACTA2	1.9*
MYL9	1.9*
MYH11	2.4*
CHRND	3.4*
ACTC1	7.2*

Figure 3.4 Diagram of the calcium signaling pathway associated with development of PSE turkey. The pathway, suggested by the IPA, showed interactions among differentially expressed genes associated with regulation of Ca^{2+} concentration between normal and PSE turkey skeletal muscle. FC, fold change or gene expression in PSE relative to normal samples. Negative FC indicates down regulation of gene in PSE samples; positive indicates up-regulation in PSE samples. *FDR<0.05.

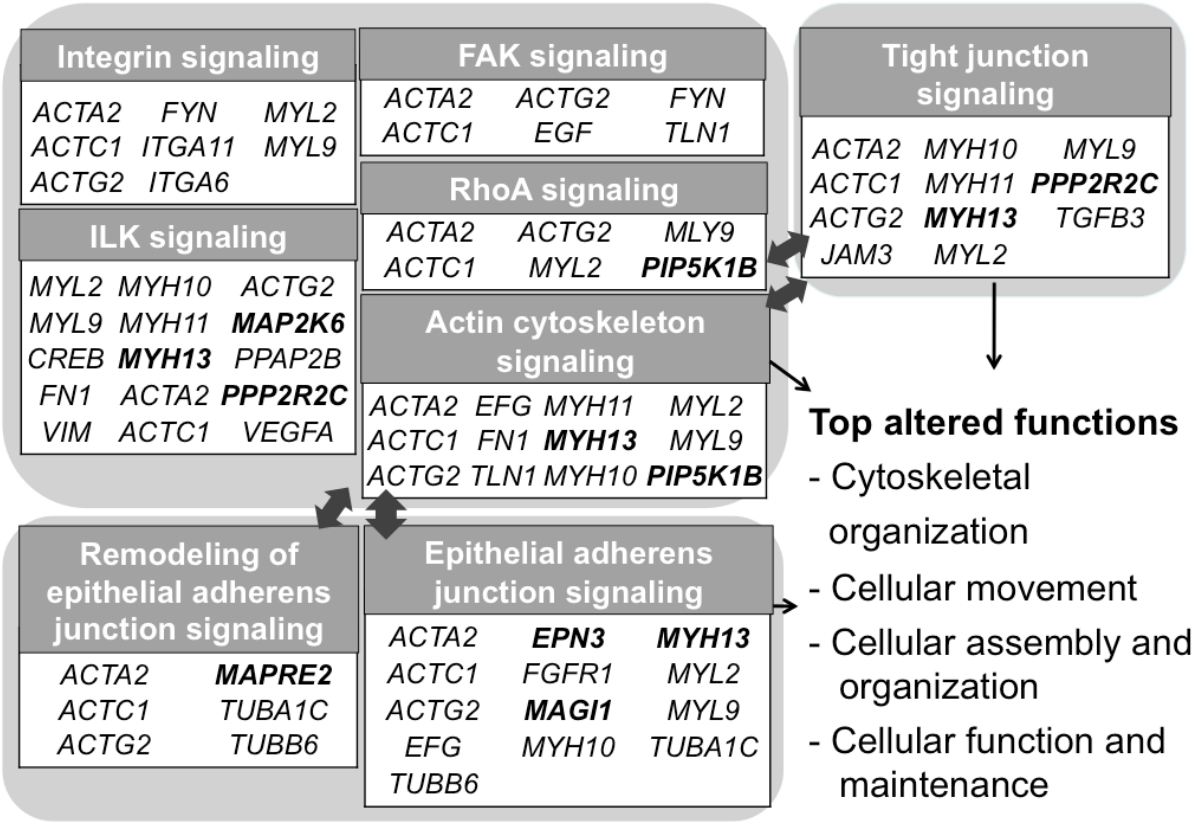


Figure 3.5 Diagram indicating overlapping canonical pathways that regulate actin cytoskeleton and cell motility, and their associated differentially expressed genes (FDR<0.05). Genes in bold show down-regulation in PSE turkey. (A) Integrin-mediated signaling pathways modulated cellular activities and signal transduction upon interactions of integrin, integrin-linked kinase (ILK), focal adhesion kinase (FAK), Ras homology family member A (RhoA) and actin cytoskeleton signaling pathways; (B) Tight junction signaling pathway interacted with RhoA and actin cytoskeleton signaling pathways and modulates tight junction complex; and (C) Epithelial-associated signaling pathways linked to actin cytoskeleton signaling, leading to actin reorganization and adheren junctions formation. Together, alteration of this signaling pathway network affects biological functions in PSE turkey.

Table 3.5 Potential downstream activities of the top altered biological functions associated with differential gene expression in PSE turkey.

Biological function	Potential activation[1]	Activation z-score[2]	Number of molecules
Cellular movement	Increased	3.34	99
Cellular assembly and organization			
- Rearrangement of cytoskeleton	Increased	2.20	8
- Growth of plasma membrane projections	Increased	1.98	25
- Microtubule dynamics	Increased	1.76	44
Cellular Function and maintenance			
- Formation of cellular protrusions	Increased	2.38	37
Energy production			
- Carbohydrate metabolism	Increased	1.76	28
- Fatty acid metabolism	Increased	1.60	28

[1] An increase or decrease in a biological process was predicted by Ingenuity Pathway Analysis (IPA) based on experimentally derived relationships between genes or proteins and directional change in expression of genes in the dataset.

[2] Activation z-score was calculated by IPA. The greater z-score implies the higher possibility of increasing activity.

Immunoblot of PDK4 protein

Upon identification of differentially expressed genes, *PDK4* was clearly of particular interest due to its substantial down-regulation in PSE samples observed in both RNA-Seq and the TSKMLO microarray analyses (Malila et al., 2013). The association of decreased *PDK4* expression and altered glucose oxidation in PSE turkey was proposed by Malila et al. (2013). Additionally, pathway analysis of the current RNA-Seq analysis emphasized aberrant energy metabolism arising from differential gene expression in PSE turkey based on the following observations. First, energy production was highlighted as the first network identified by IPA. Second, based on the direction of differential gene expression, IPA predicted an increase in carbohydrate and lipid metabolic activities in PSE samples (Table 3.5), which is consistent with the hypothesis of hypermetabolism in the susceptible turkeys. Taken together, *PDK4* was prioritized in this study for further investigation at the translational level.

Abundance of PDK4 protein in turkey breast meat was determined using protein immunoblot assay (Figure 3.6). The results demonstrated that PDK4 protein was 3.4 fold lower in PSE meat compared to normal samples ($P < 0.001$).

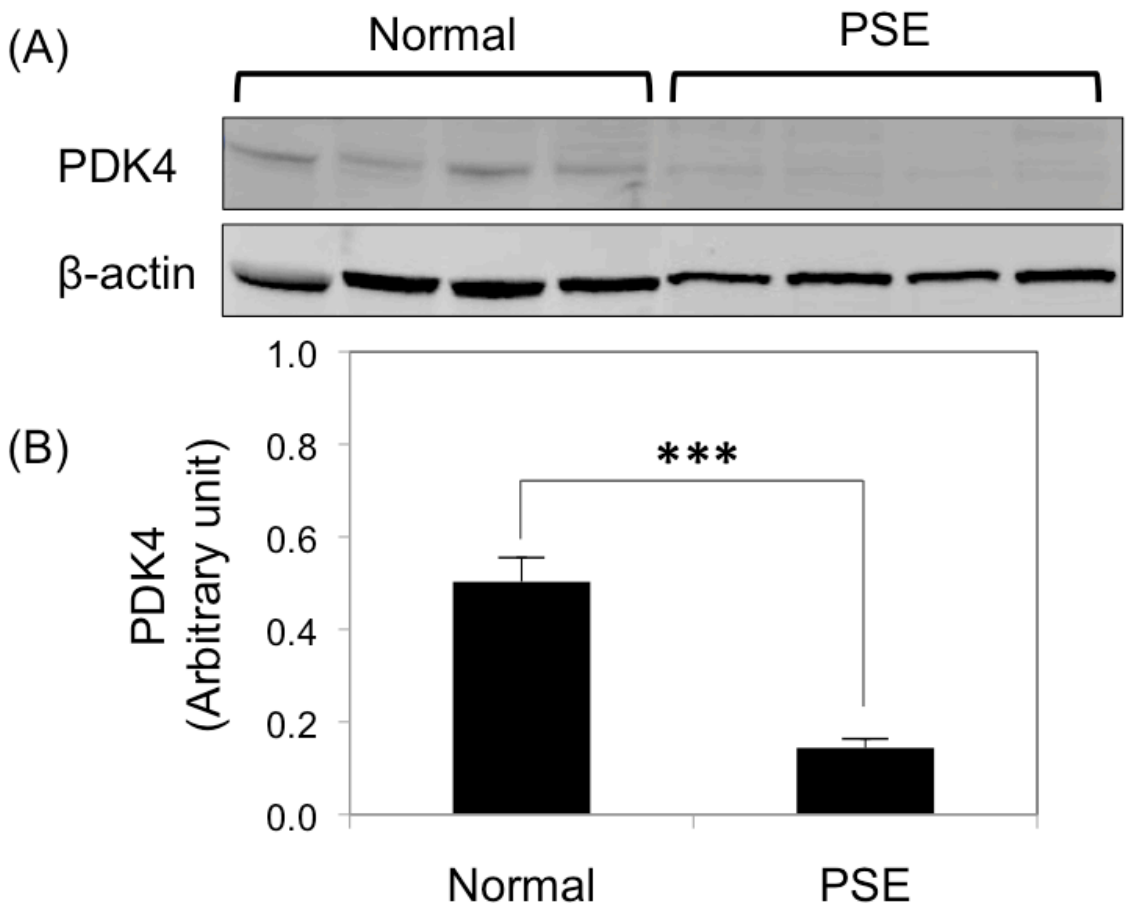


Figure 3.6 Expression of PDK4 protein in turkey breast muscle. (A) Protein immunoblot shows PDK4 protein abundance in normal (n = 4) and PSE (n = 4) turkey meat. Each lane presents analysis of proteins collected from an individual bird. Beta-actin was used as an endogenous control. Blot is a representative from three technical replicates. (B) Histograms, constructed from densitometry of immunoblot results, represent difference in abundance of PDK4 protein expressed in normal and PSE turkey. Expression of PDK4 protein is presented as ratio of band intensity between PDK4 and β-actin within lane. Average expression of PDK4 was calculated from results of three different blots. $^{***}P < 0.001$

3.4 Discussion

Previous studies of differential gene expression regarding development of PSE turkey were mainly focused on individual SR Ca^{2+} regulators (Chiang et al., 2004; Chiang et al., 2007; Oda et al., 2009; Sporer et al., 2012). However, the results suggested that these single-gene approaches were inadequate in addressing the evident complex etiology of this meat defect. Recently, a microarray specifically developed to analyze gene expression in turkey skeletal muscle was used to profile the transcriptomes of PSE and normal turkey breast meat samples (Malila et al., 2013). Differential expression of many genes and interactions among molecular signaling pathways were observed which offered new insights into the mechanism of PSE meat development.

In the current study, the Illumina GA_{IIX} RNA-Seq platform was utilized to complement and extend our previous microarray study. The RNA-Seq analysis revealed 494 differentially expressed transcripts (FDR < 0.05) and multiple associated signaling pathways associated with development of PSE turkey. Despite the inherent advantages of RNA-Seq over microarray, we also experienced some challenges in RNA-Seq data analysis. Some differentially expressed transcripts identified in the microarray study (Malila et al., 2013) were classified in this RNA-Seq analysis with a low priority Cufflinks class code, or were not present in the current turkey genome assembly. Because the turkey reference genome is still an early draft, these results are not surprising. In addition, some large gene families such as myosin comprise closely related individual genes that are not yet well annotated in

the turkey reference genome; thus, loci generated from the RNA-Seq read alignments against these reference genes are not well defined. In contrast, probes on the TSKMLO microarray were designed based on turkey skeletal muscle cDNA libraries constructed and annotated with the chicken genome because the turkey reference genome was not available (Reed et al., 2008). Since the chicken genome is more established, the TSKMLO microarray study may identify genes that are not yet annotated in the turkey reference genome (Malila et al., 2013). However, the RNA-Seq data can be realigned with updated versions of the turkey reference genome as new versions become available in the future.

The current RNA-Seq analysis confirmed the important roles of actin cytoskeleton signaling, RhoA signaling, and the calcium signaling pathways associated with development of PSE turkey. The findings extended interactions within and between actin cytoskeleton and RhoA pathways to a larger network of integrin-related signaling pathways (Figure 3.5), supporting the role of irregular actin cytoskeletal filaments in PSE turkey. The turkey PSE problem is clearly multifactorial with respect to the involvement of various genes and molecular pathways. The hypothesis of fast-to-slow skeletal muscle isoform conversion in PSE turkey, originally proposed in the microarray study, was supported in this study by down-regulation of fast-switch myosin heavy chain (**MYH**; *MYH13*, FC = -5.0), and up-regulation of slow-switch isoforms (*MYH10*, FC = 1.5; *MYH11*, FC = 2.4) in PSE samples.

For the calcium signaling pathway, a greater mRNA abundance of the nicotinic cholinergic receptor gene (***CHRND***) was observed in PSE turkey (FC =

3.4). This gene encodes the delta subunit of the muscle acetylcholine receptor (**AchR**). In skeletal muscle, the acetylcholine receptor, a pentameric protein composed of five subunits with the stoichiometry $\alpha_2\beta\gamma\delta$, is located at the neuromuscular junction (Goldman et al., 1988). In neuromuscular signal transduction, acetylcholine binds to the receptor, which then undergoes a conformational change, leading to opening of an ion-conducting channel across the plasma membrane (Pedersen and Cohan, 1990). A mutation of *CHRND* has been implicated in functional disruption of acetylcholine receptor, leading to an abnormality of muscle contraction (Engel and Sine, 2005). Activity of this receptor requires coordination of all subunits; thus, the increased expression of *CHRND* in PSE samples may affect the function of acetylcholine receptor and alter ionic homeostasis, including Ca^{2+}, in the skeletal muscle cell. This is the first evidence indicating that aberrant Ca^{2+} regulation in PSE turkey may involve a plasma membrane Ca^{2+} channel.

Malila et al. (2013) reported a substantial down-regulation of *PDK4* in PSE turkey. This gene encodes an enzyme that catalyzes phosphorylation of the alpha subunit of mitochondrial pyruvate dehydrogenase complex (**PDH**) into an inactive form, thus inhibiting conversion of pyruvate into acetyl CoA (Popov, 1997; Wynn et al., 2008). Based on the previous findings, we hypothesized that down-regulation of *PDK4* may result in a decreased level of PDK4 enzyme in the susceptible animals. Therefore, at the early-stage of postmortem muscle metabolism, the flux of conversion of pyruvate into acetyl CoA by PDH would be greater in PSE samples.

Oxygen depletion may be faster. Thus, the metabolism switches from oxidative to anaerobic faster in the susceptible animals, leading to a rapid pH drop (Figure 3.7). High acidity combined with high carcass temperature at the early postmortem-stage is associated with protein denaturation in the defective turkey meat. Although a large fold-change of *PDK4* was observed in the microarray study (Malila et al., 2013), the change was not statistically significant. Herein, RNA-Seq revealed differential expression of *PDK4* (FC = -14.1) at FDR < 0.05. Immunoblot assays of PDK4 protein (Figure 3.6) also demonstrated low abundance of PDK4 in PSE samples ($P < 0.001$). This result strongly supports the hypothesis of dysregulation of postmortem oxidative glucose metabolic pathways.

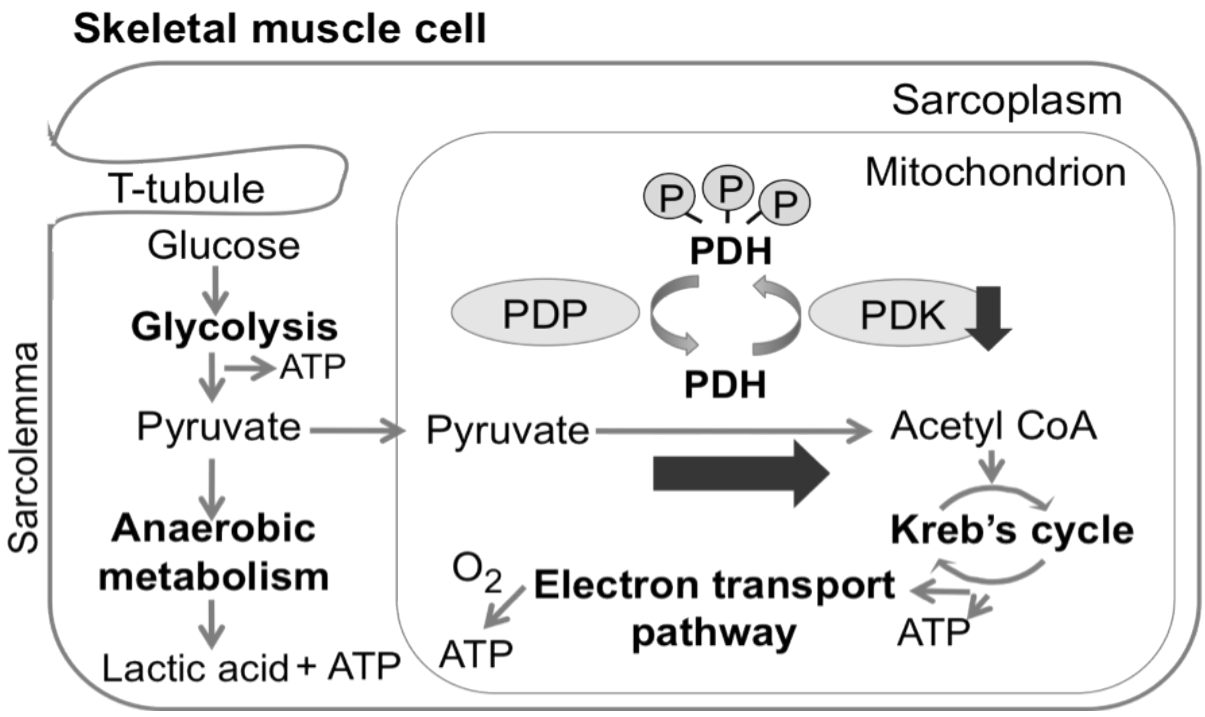


Figure 3.7 Schematic diagram representing mitochondrial oxidative metabolism altered by decreased expression of *PDK4* in PSE turkey. Mitochondrial pyruvate dehydrogenase complex (PDH) is regulated by pyruvate dehydrogenase phosphatase (PDP) or PDK4, thus contributing to the regulation of glucose metabolism. Down-regulation of *PDK4* gene expression in PSE turkey may alter regulation of conversion of pyruvate to acetyl CoA and accelerate early stage postmortem oxidative metabolism.

In the current study, pathway analysis revealed altered metabolic processes of not only carbohydrate, but also lipid. Based on the role of *PDK4* in regulating energy production (Figure 3.3), *PDK4* may cross-communicate between glucose and fatty acid metabolisms. Interactions between glucose and fatty acid metabolisms in

muscle and adipose tissue have been proposed by Randle et al. (1963) as a glucose fatty-acid regulatory cycle. To maintain glucose level during starvation, glucose oxidation is switched off through phosphorylation of PDH by PDK4, and fatty acids are used as fuel in production of acetyl CoA and NADH (Randle et al., 1994). Sugden et al. (2001) also proposed that PDK4 acts as a mediator for fatty acid oxidation by increasing available pyruvate for oxaloacetate formation. In a study on differential gene expression in chicken breast muscle, Sibut et al. (2011) reported down-regulation of *PDK4* in high-glycogen breast samples compared with low-glycogen samples. In contrast, when comparing high-fat traits relative to low-fat ones, they observed up-regulation of *PDK4*, suggesting a strong interaction between carbohydrate and lipid metabolism. However, it must be noted that in the study of Sibut et al., chicken muscle samples were classified based on glycogen content or fat content to determine the molecular mechanisms involved in variation of meat quality. In this study, the samples were first classified as PSE or normal, then analyzed and compared for differential gene expression.

The change in expression of *PDK4* may alter contraction and relaxation process of skeletal muscle. Recently, Herbst et al. (2012) observed a significant increase in initial force of *PDK4*-knock-out mouse skeletal muscle in response to moderate-intensity stimulation. This leads us to hypothesize that abnormal Ca^{2+} regulation combined with down-regulation of *PDK4* may provide an effect on extensive muscle contraction in PSE-susceptible birds that accelerates early-stage postmortem metabolism.

As in other transcriptome analyses, RNA-Seq depicts steady state transcript abundance which can be utilized to monitor changes in gene expression in response to biological stimuli. Such analyses reveal candidate gene sets that with further validation can subsequently be used for classification of samples. Although transcriptional level changes generally correspond to changes in translation, mRNA abundance does not always directly reflect expression of encoded proteins (Preiss et al., 2003). To gain a complete understanding of molecular mechanisms underlying development of PSE turkey, a series of studies must be completed for candidate genes at the protein level, including protein abundance and protein activity. In this study, we provided an example of a protein level investigation by performing a PDK4 immunoblot. Decreased PDK4 protein abundance in PSE meat corresponded to decreased transcript abundance. The results support the important role of PDK4 in development of PSE turkey.

3.5 Conclusions

Study of global differential gene expression between normal and PSE turkey using RNA-Seq technique highlighted complex interactions of multiple cellular signaling pathways associated with differential gene expression in development of PSE turkey. The results from the current RNA-Seq complement and extend the previous findings from the microarray analysis. Difference in mRNA abundance of nicotinic acetylcholine receptor delta subunit suggests, for the first time, that aberrant calcium homeostasis may be based not only at the SR level, but also extracellular calcium regulation. Alteration of cellular signaling pathways associated with organization of actin cytoskeleton has been confirmed and the complexity of the

network has been extended. Substantial down-regulation of PDK4 at transcriptional and translational levels in PSE turkey supports the hypothesis of an abnormality in glucose oxidative pathway, which may result in an unusually high rate of postmortem metabolism in PSE turkey. Activities of PDK4 in PSE turkey must be further investigated for better understanding of its mechanisms underlying development of PSE turkey. The results also suggest PDK4 as a potential gene marker to identify susceptible turkeys from the population.

CHAPTER 4

EXPRESSION OF PYRUVATE DEHYDROGENASE KINASE ISOZYME 4 IN PALE, SOFT AND EXUDATIVE MEAT FROM RANDOMBRED AND MODERN COMMERCIAL TURKEYS

ABSTRACT

Fundamental mechanisms responsible for post-mortem hypermetabolism associated with development of PSE turkey remain unclear. Previous transcriptome analyses of turkey breast muscle from the randombred control line (RBC2), representative of turkeys from the 1960s maintained without selection pressure, revealed substantial down-regulation of the pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) gene in PSE samples. PSE meat quality continues to be a problem in modern commercial (COMM) turkey lines genetically selected for increased breast muscle mass. We hypothesized that as in the RBC2 line, PDK4 is also down-regulated in PSE turkey from COMM lines and that decreased *PDK4* transcript levels causes decreased PDK4 protein abundance. The objective of this study was to determine transcript and protein levels of PDK4 in PSE and normal turkey muscle within both COMM and RBC2 lines. Turkey breast muscle samples were harvested from both lines (RBC2, n = 43; COMM n = 39), and meat quality characteristics were determined. Breast samples were classified as normal or PSE based on marinade uptake (high = normal; low = PSE). Turkey breasts from each line with the highest (n = 6) and lowest (n = 6) marinade uptake were utilized as normal and PSE samples. Change in expression of PDK4 was determined within line. Transcript abundance of *PDK4* mRNA was quantified using quantitative real-time PCR. Significant down-regulation of *PDK4* was observed in PSE meat of both lines ($P < 0.05$) with a 4.3-

fold decrease in RBC2 and a 12.8-fold decrease in COMM turkey. Microsomal protein was prepared from the normal and PSE meat samples from each line and subsequently utilized for determination of PDK4 protein using immunoblotting assay. Decreases in PDK4 protein level were found with high significance ($P < 0.001$) in both lines (RBC2, FC = -3.4; COMM, FC = -2.6) and in agreement with transcript differences. The results support the biological relevance of PDK4 suppression in the development of PSE turkey meat, and also suggest that the mechanism responsible for the decreased PDK4 in RBC2 turkey subpopulations has been maintained in a commercial line.

4.1 Background

Over the past several decades, the turkey breeding industry has worked to meet increasing consumer demand for poultry products by improving turkey growth rate and carcass muscle mass. Compared to the commercial turkeys of the 1960s, modern commercial turkeys are marketed in about half the time (reviewed by Barbut et al., 2008) and yield heavier breast muscle mass (Fernandez et al, 2001; Lilburn and Nestor, 1991; Updike et al., 2005). However, as a consequence of the successful turkey-breeding selection, the pale, soft and exudative (**PSE**) meat defect in turkey has become more prevalent (Dransfield and Sosnicki, 1999). Processed meat products made from PSE meat are typically inferior because of reduced protein functionality, including reduced solubility, reduced binding, and poor gelation properties. This problem poses an increasing challenge to processing plants as consumer preferences trend toward value-added, processed meat products instead of whole bird or bird cuts.

Development of PSE turkey is associated with an accelerated postmortem glycolysis that leads to rapid pH decline in the muscle cell (Pietrzak et al., 1997). It has been hypothesized that changes in such biochemical conditions of PSE turkey associated with intracellular [Ca^{2+}] overload due to a leak by sarcoplasmic reticulum calcium channel proteins that promotes hypermetabolism of skeletal muscle. Recently, transcriptome profiling studies of normal and PSE turkey breast meat collected from the randombred control line (**RBC2)**, a non-selected line representing commercial turkeys of the 1960s (Nestor et al., 1967; Nestor, 1977a; Nestor, 1997b), indicated differential expression of genes involved in Ca^{2+} regulation in muscle cells, supporting the hypothesis of an abnormality in calcium homeostasis in PSE turkey (Malila et al., 2013; Malila et al., in preparation). Additionally, the studies revealed changes in expression of multiple genes from several cellular signaling pathways, including energy production and actin cytoskeletal organization, as well as interactions among the associated pathways, indicating the complexity of development of PSE turkey.

One finding of particular interest is down-regulation of the gene encoding pyruvate dehydrogenase kinase isozyme 4 (***PDK4***) identified in both microarray (fold change, **FC** = -25.9) (Malila et al., 2013) and deep transcriptome sequencing (**RNA-Seq**; FC = -14.1) platforms (Malila et al., in preparation). This enzyme inactivates the pyruvate dehydrogenase complex (**PDH**) through phosphorylation of serine residues located on $E_1\alpha$ subunit of PDH, thus inhibiting conversion of pyruvate to acetyl CoA during glucose catabolism (Sugden and Holness, 2003). In concert with decreased

transcript abundance, there was a significant reduction of PDK4 protein abundance in PSE turkey from the RBC2 line (Chapter 3). Decreased expression of PDK4 at both transcriptional and translational levels in PSE meat from random-bred turkey suggests that an acceleration of early-stage postmortem metabolic rate in the susceptible birds may not only derive from accelerated glycolysis, but may also include an altered glucose oxidative metabolism.

The determination of PDK4 expression was previously limited to a small subset of normal and PSE samples from RBC2 turkey. The objective of this study was to assess differences in PDK4 transcript and protein abundance within a larger RBC2 turkey sample size and to determine whether these differences persist within an intensively selected, modern commercial line.

4.2 MATERIALS AND METHODS

Sample information

Turkey breast meat was a subset of meat samples collected in the study of Chiang et al. (2008). In brief, RBC2 turkeys were obtained from The Ohio Agricultural Research and Development Center of The Ohio State University. Growth-selected COMM turkeys were obtained from a local turkey producer. The turkeys from RBC2 line (43 birds) were harvested at 22 weeks of age while COMM birds (39 birds) were harvested at 16 weeks of age (Chiang et al., 2008). Breast muscle samples were collected, immediately snap frozen in liquid nitrogen and stored at -80°C for RNA isolation and microsomal protein preparation. Meat quality

indices of the samples were determined in the study of Chiang et al. (2008). The samples were classified as normal or PSE primarily based on percent marinade uptake (high = normal, low = PSE) as previously described (Malila et al., 2013; Sporer et al., 2012). Six samples for each extreme of normal and PSE characteristics (n = 6) from each line were used in this study.

RNA Isolation and quantitative real-time PCR (qPCR)

Total RNA of RBC2 samples were the same materials isolated and utilized in the previous transcriptome analyses (Malila et al., 2013; Malila et al., in preparation). Total RNA was isolated from breast meat samples in accordance with the protocol previously described (Malila et al., 2013). Quantity of total RNA was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Integrity of total RNA was confirmed using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Samples with an RNA Integrity Number (**RIN**) equal to or exceeding 8.0 (RIN = 10 is the best) were used in qPCR.

Primers were designed using Primer Express 3.0 software (Applied Biosystems) and synthesized by Operon Inc. (Huntsville, AL). Primer sequences were the following: *PDK4* (GenBank# NM_001199909) forward $5'_{3347}$-ATGAATGTCTGTAATAGTGCTTGCAA-$3'_{3472}$ and reverse $5'_{3536}$-CATGTCTTCATTGTATGTTCTGCATATAC-$3'_{3508}$ with a product size of 90 bp; *ACTB* (β-actin, GenBank# AY942620) forward $5'_{1071}$-GTCCACCTTCCAGCAGATG TG-$3'_{1091}$ and reverse $5'_{1132}$-CAATGGAGGGTCCGGATTC-$3'_{1111}$ with a product

size of 71 bp. The qPCR protocol was described in Malila et al. (2013). Beta-actin was used as an endogenous control gene (Sporer et al., 2012). Expression of *PDK4* in PSE samples relative to normal samples was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Microsomal membrane preparation

Microsomal membrane samples were prepared as previously described (Malila et al., in preparation). All preparation steps were carried out at 4°C. Concentration of crude microsomal protein was determined using the Pierce Bicinchoninic acid (**BCA**) Protein Assay kit (Thermo Scientific, Rockford, Illinois) following manufacturer's recommendation.

Protein electrophoresis and immunoblot assay

Electrophoresis and immunoblot analysis were conducted according to the procedure described in Malila et al. (in preparation). Briefly, crude microsomal proteins were separated by electrophoresis using a 12% Tris-glycine Criterion® gel with dimension of 133 x 87 x 1 mm (Bio-Rad, Hercules, CA) at constant 200 V for 1 hr at 4°C. The gel was subsequently equilibrated in 100 mL transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 10 min at room temperature and blotted with methanol-prewetted Immobilon-FL polyvinylidene difluoride (**PVDF**) membrane (Millipore, Burlington, MA) using a Criterion blotting cell (Bio-Rad). The transfer of proteins was conducted at constant 30 V for 4 h at 4°C. After blotting, the

membrane was briefly washed with Tris-saline-tween buffer (**TBST**) for 5 min and blocked with 20 mL of the LI-COR blocking buffer (LI-COR, Lincoln, NE) for 1 h at room temperature. The membrane was then incubated overnight at 4°C with a mixture of primary antibodies (1:500 polyclonal rabbit Anti-PDK4 (101-114) antibody, Sigma-Aldrich, and 1:10,000 monoclonal mouse anti-β-actin antibody, Sigma-Aldrich). The blot was washed with TBST for 5 min at room temperature, repeating 4 times and then incubated with a mixture of secondary antibodies (1:3000 anti-rabbit IgG IRDye 700DX® conjugated antibody, 1:3000 anti-mouse IgG IRDye800® conjugated antibody, Rockland, Gilbertsville, PA) in dark for 1 h at room temperature. The membrane was scanned using the Odyssey® Infrared Imaging System (LI-COR).

Image analysis

Immunoblot image analysis was carried out as previously described in Chapter 3. In brief, densitometry was performed using ImageJ 1.46r (Rasband, 2012). A rectangular box was manually placed around the band of interest in the first lane. Features with identical size were used to measure the other bands for all lanes. Band intensity of PDK4 was normalized with the in-lane intensity value of β-actin, and the ratio values was defined as protein abundance. B-actin was used as a control.

Because the assay was conducted in triplicates, there were three ratio values, obtained from different blots, per biological replicate. Average of the three ratio values was used as protein abundance of each bird in statistical analysis.

Statistical analysis

Statistical analysis in expression of PDK4 between normal and PSE turkey within genetic line was assessed using Student's t test. Significant differences were identified at $P < 0.05$.

4.3 Results

Meat quality indices of normal and PSE turkey meat

Meat quality indices of normal and PSE turkey meat are shown in Table 4.1. The PSE meat from both lines exhibited significantly reduced marinade uptake and increased cook loss compared with normal samples ($P < 0.05$). Significant differences in 15 min postmortem pH and L^* value were found only in COMM turkeys. Drip loss was not statistically different within either turkey genetic line.

Table 4.1 Meat quality indices of the meat samples utilized in the study of PDK4 expression in RBC2 and COMM turkey.

Meat quality index[1]	RBC2[2]		COMM[2]	
	Normal	PSE	Normal	PSE
pH_{15}	5.72 ± 0.11	5.52 ± 0.08	6.02 ± 0.09[a]	5.77 ± 0.05[b]
L* value	54.2 ± 0.65	56.2 ± 0.97	57.9 ± 0.54[b]	61.0 ± 0.86[a]
% Marinade uptake	45.3 ± 4.65[a]	17.2 ± 1.02[b]	25.9 ± 2.62[a]	7.3 ± 1.30[b]
% Drip loss	0.52 ± 0.10	0.68 ± 0.16	0.55 ± 0.35	1.44 ± 0.38
% Cook loss	27.8 ± 0.84[b]	30.7 ± 0.32[a]	28.2 ± 0.48[b]	32.1 ± 0.62[a]

[1] Meat quality was determined in the study of Chiang et al. (2008). The results shown here are specific for the meat samples defined as normal (n = 6) or PSE (n = 6) that were utilized in this study. The results are presented as mean ± SEM. The pH was determined at 15 min postmortem. The other indices were determined at 24 h postmortem. Different superscript letters indicate significant difference ($P < 0.05$) between normal and PSE samples within genetic line.

[2] RBC2 = randombred control line, COMM = commercial line

Expression of PDK4 at transcriptional and translational levels in turkey meat

Differences in mRNA abundance of *PDK4* were observed in PSE turkey samples collected from both RBC2 and COMM lines (Figure 4.1). In RBC2 turkey, *PDK4* was down-regulated by -4.3 fold ($P < 0.05$), whereas expression of *PDK4* in PSE meat from COMM line was reduced -12.8 fold ($P < 0.05$).

A decrease in PDK4 protein abundance in PSE turkey meat from both lines was observed in agreement with transcript difference (Figure 4.2). Reduced PDK4 protein showed high significance ($P < 0.001$) in both lines (RBC2, FC = -3.4; COMM, FC = -2.6).

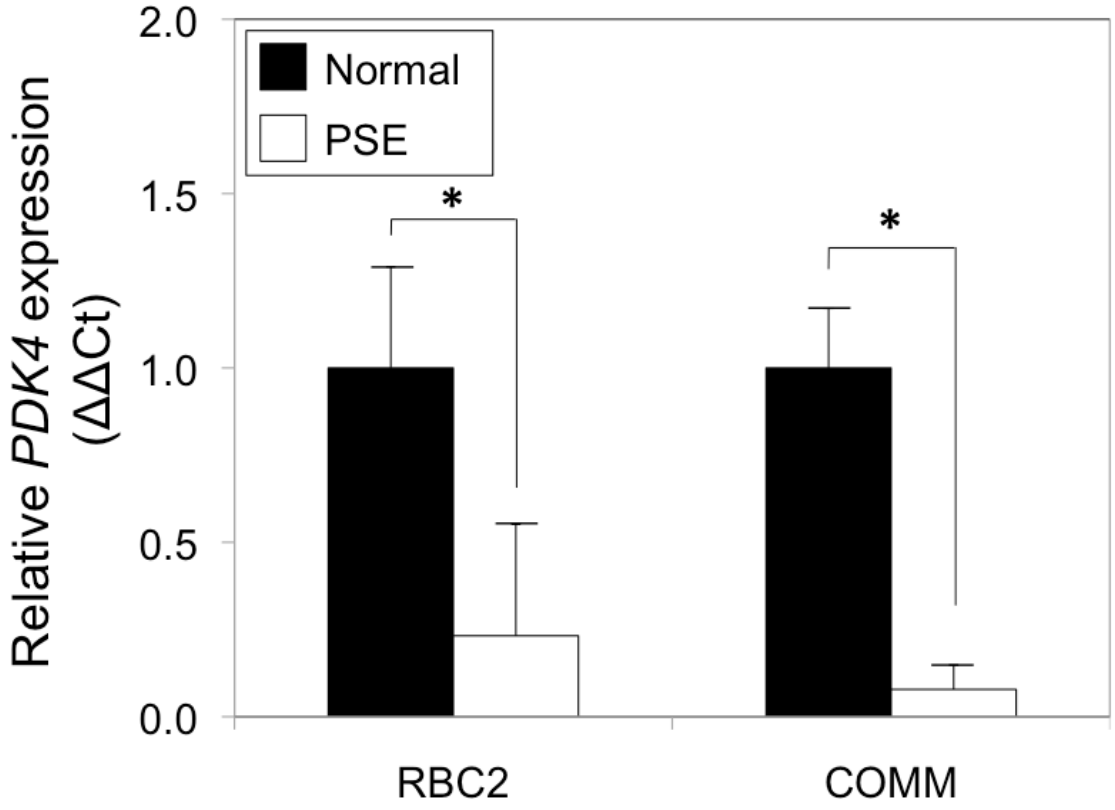


Figure 4.1 Relative mRNA abundance of *PDK4* between normal and PSE turkey as determined using qPCR. Bars represent mean ΔΔCt ± SEM of PSE relative to the normal sample within line. B-actin was used as an endogenous control. Expression of *PDK4* decreased 4.3-fold in PSE samples of RBC2 line (n = 6) and 12.8-fold in the PSE samples of COMM line (n = 6). *$P < 0.05$.

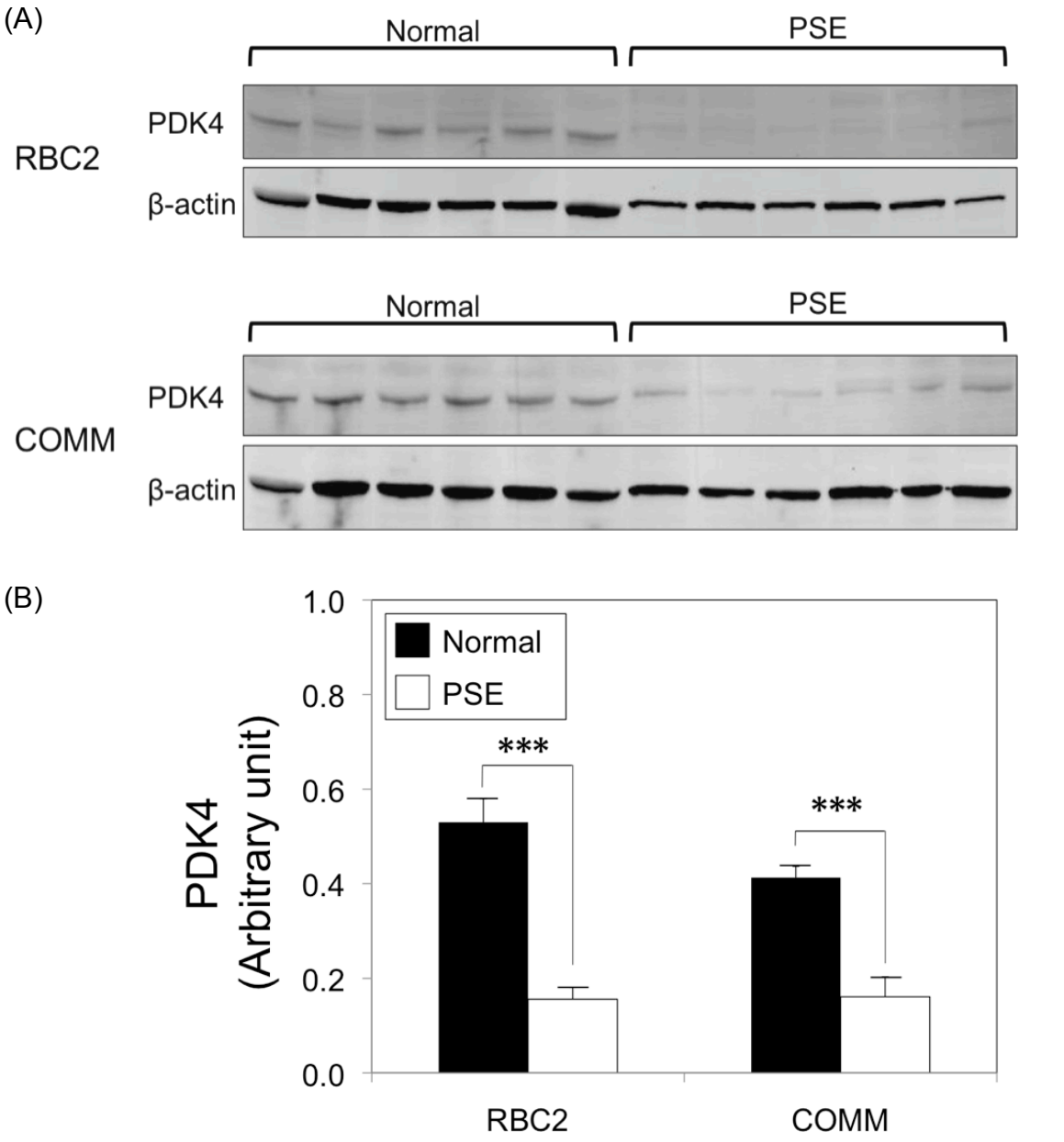


Figure 4.2 Differences in abundance of PDK4 protein between normal and PSE turkey. (A) Immunoblots of PDK4 between normal (n=6) and PSE (n=6) turkey collected from RBC2 and COMM lines. (B) Histograms show mean±SEM calculated from band intensity of PDK4 normalized with intensity value of β-actin from the same lane. Statistical analysis was performed within genetic line. ***P < 0.001

4.4 DISCUSSION

In response to high consumer demand for poultry products, turkey breeding programs have focused on enlarging muscle mass while enhancing growth rate. However, the success of the breeding program has coincided with the increasing prevalence of undesirable traits, including myopathies and skeletal deficiencies, which eventually have a detrimental effect on meat quality (Dransfield and Sosnicki, 1999). In this study, it is notable that marinade uptake of breast meat from modern growth-selected COMM turkeys was lower than that of random-bred RBC2 line regardless of normal or PSE meat classification. This evidence corresponds with previous reports indicating that turkey breeding selection program concentrating only on accelerated growth rate and muscling mass consequently worsens turkey meat quality (Dransfield and Sosnicki, 1999; Updike et al., 2005).

The problem of PSE turkey was first documented in the 1970s (Vanderstoep and Richards, 1974; van Hoof, 1979). Development of this turkey meat defect is associated with postmortem hypermetabolic rate in the susceptible birds (Vanhoof, 1979; Pietrzak et al., 1997). In this study, no significant difference in pH at 15 min postmortem between normal and PSE turkey was observed in the RBC2 birds. However, this parameter of PSE samples from COMM line was significantly lower than that of normal samples ($P < 0.05$). The results suggest high rate of early postmortem metabolism in the susceptible birds from the modern line.

According to the previous global gene expression analyses (Malila et al., 2013; Malila et al., in preparation), substantial down-regulation of *PDK4* was found in PSE meat from RBC2 line. Due to its roles in regulating glucose catabolism, this gene was of particular interest. We hypothesized that the lower *PDK4* mRNA abundance in PSE meat may result in a decreased level of PDK4 enzyme. The change in amount of this enzyme may alter glucose oxidation and postmortem metabolic rate in the PSE turkey. In this study, the previous hypothesis is supported by the immunoblot assay showing that, in RBC2 samples, PDK4 protein was expressed 3.4-fold lower in PSE meat compared to normal samples ($P < 0.05$).

The initial analysis of global gene differences between normal and PSE meat was conducted in RBC2 turkeys maintained without selection pressure, to serve as a basis for understanding the role of genetics of PSE development in modern growth-selected turkeys. In this study, expression of PDK4 was compared between normal and PSE turkey within genetic lines, RBC2 and COMM lines. For RBC2 samples, the sample size was expanded from the previous immunoblot analysis (Malila et al., in preparation) to cover all RBC2 samples utilized in the microarray study (Malila et al., 2013). Down-regulated expression of *PDK4*, at both transcriptional and translational levels, was clearly observed in PSE meat of both RBC2 and COMM line ($P < 0.05$). Reduced expression of PDK4 in both lines strongly confirms the biological relevance of PDK4 dysregulation in PSE turkey. The results also suggest that the decrease in

PDK4 expression has been maintained during breeding selection of the COMM turkeys, in which the problem of the PSE defect appears to be more pronounced.

It can be speculated that, at the early postmortem stage, when oxygen is still present, glucose undergoes aerobic catabolism in order to generate ATP for cellular activities. However, due to the reduction of PDK4 level in the PSE-susceptible turkeys, PDH continues converting pyruvate into acetyl CoA without the inhibiting signal from PDK4, resulting in accelerated rate of glucose oxidation. Oxygen is rapidly consumed. Metabolism switches from aerobic into anaerobic pathways faster in the susceptible birds, resulting in greater amounts of lactic acid accumulated in the muscle cell that causes rapid postmortem pH decline in the defective meat. In addition, as increased ATP may be generated in the susceptible turkeys, H^+ production during hydrolysis of ATP may accelerate pH drop and augment the acidosis in the susceptible birds.

Based on the hypothesis of altered glucose oxidation in PSE turkey, ATP levels in PSE turkey may be greater than that of normal samples as ATP is continuously generated via glucose oxidation early postmortem, and rapidly utilized for cellular activities afterward. Rapid ATP degradation was previously observed in skeletal muscle of pigs carrying heterozygous halothane gene, the gene associated with lean muscle, but pigs carrying this gene potentially yield PSE meat (Lahucky et al., 2002; Moesgaard et al., 1995; Shen et al., 2007). However, in those studies, the ATP content at the beginning of the time-course experiments was not significantly different between halothane-gene carriers and

non-carriers. In addition, Miri et al. (1992) and Batlle et al. (2000) reported lower postmortem ATP content in PSE pork. However, Miri et al. and Batlle et al. quantified ATP at 30 min postmortem and 2h postmortem, which might be too metabolically advanced and thus they were not able to capture the rapid ATP metabolism at the onset of postmortem conversion of muscle to meat. Comparison of ATP content and its degradation rate between normal and PSE turkey at the onset of postmortem will provide insight regarding the impact of down-regulated PDK4 on glucose oxidative metabolism.

Knowledge of the mechanisms regulating PDK4 expression is beneficial for developing pathway intervention approaches or nutritional modification to minimize the incidence of PSE turkey. Expression of *PDK4* is suppressed by insulin and induced by glucocorticoids (Connaughton et al, 2010) as well as high-fat feed (Holness et al., 2000). Increase in PDK4 protein level was also found during starvation (Wu et al., 2000). Above all, better understanding of PDK4 roles on development of PSE meat defect is needed so that the most effective approaches can be carefully developed and implemented without creating other problems for the industry in the future.

4.5 Conclusions

A significant decrease in PDK4 mRNA and protein was identified in both modern and random-bred turkey lines. This observation supports the hypothesis of an abnormality in the glucose oxidative pathway, which may result in unusually high rate of postmortem metabolism in PSE turkey. Comparison of PDK4 and

PDH activity, as well as oxygen consumption rate, between normal and PSE turkey will provide better confirmation of the hypothesis of glucose oxidative dysregulation and better comprehension on PDK4 biological roles on development of this turkey meat defect.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Differential gene expression between turkey skeletal muscle samples of normal and PSE turkey meat collected from the randombred control line (**RBC2**) has been revealed by transcriptome analyses. In the global gene expression analysis using the turkey skeletal muscle long oligonucleotide (**TSKMLO**) microarray, 49 transcripts were reported with significant difference in expression at FDR < 0.1. Deep transcriptome sequencing analysis (**RNA-Seq**) identified 494 differentially expressed transcripts between normal and PSE samples at FDR < 0.05. Pathway analysis strongly supports the association of multiple cellular mechanisms in development of the PSE turkey meat defect.

A clear association between an abnormality of Ca^{2+} homeostasis and development of this turkey meat defect has been confirmed. In addition, up-regulation of the gene encoding the delta subunit of the skeletal muscle nicotinic acetylcholine receptor was identified in PSE turkey, suggesting dysregulation of $[Ca^{2+}]$ in PSE turkey at both intracellular and extracellular levels. Actin cytoskeletons of the PSE-susceptible birds are affected as multiple signaling pathways regulating formation and reorganization of actin cytoskeleton are altered. Irregular actin cytoskeleton-related pathways might affect various downstream cellular activities as well as structural stability of muscle cells. Muscle cells of the susceptible birds may be intolerant of a drastic change of muscle shape and volume during extensive muscle contraction; hence, muscle damage,

which detrimentally affects quality of the meat. Additionally, down-regulation of genes encoding fast-twitch muscle isoforms and down-regulation of slow-twitch isoforms may imply fast-to-slow switch muscle isoform conversion. The change in ratio of muscle isoforms and the other skeletal muscle regulatory proteins may interfere with biochemical activities inside the cells. Changes in biochemical activities either from irregular actin cytoskeleton-mediated pathways or from transformation of muscle isoforms may manifest the process of conversion of muscle to meat, leading to the inferior characteristics of PSE turkey.

Based on the reduction of both mRNA and protein abundance of pyruvate dehydrogenase kinase isozyme 4 (**PDK4**) in PSE turkey from the randombred line, we propose a new postmortem paradigm regarding development of PSE turkey. The rapid postmortem pH drop in the susceptible birds may not be only from high rate of glycolysis, but also from an altered regulation of glucose oxidative metabolism. At the early postmortem stage when oxygen is still present, muscle cells generate energy for cellular activities via glucose oxidative metabolism. Subsequently, when oxygen is completely consumed, the pathways switch to anaerobic pathways in which lactic acid is produced, causing pH decline in the muscle. However, in the PSE-susceptible turkeys, a decrease in PDK4 level accelerates conversion of pyruvate into acetyl CoA. Oxygen is consumed more rapidly, leading to a faster rate of postmortem metabolic switch from aerobic to anaerobic pathways. A large amount of lactic acid accumulation combined with protons generated from ATP hydrolysis results in rapid pH decline. The biological relevance of decreased PDK4 expression and development of PSE turkey is

strongly supported by down-regulation of PDK4 transcript and protein abundance in PSE meat of the modern commercial line in which more frequent prevalence and greater magnitude of the PSE problem has been observed.

Further investigation is recommended to test the proposed hypothesis. The study may include comparison of PDK4 activity between normal and PSE turkey. This can be indirectly indicated by determining protein abundance of pyruvate dehydrogenase (PDH) and its phosphorylated forms using immunoassays. Lower phosphorylated PDH abundance is anticipated in PSE samples. In addition, a study designed to follow interval postmortem ATP change in turkey meat samples will test the hypothesis of an abnormality of glucose oxidative metabolism in PSE turkey. A set of meat samples from each biological replicate can be collected at different postmortem time-points and immediately snap frozen, hence each sample provides a snapshot of metabolic information at a definite postmortem period. It can be presumed that, once all of the time-point ATP metabolic data is combined, the dataset will depict overall postmortem ATP metabolism in turkey meat. The quantity of ATP can be determined in intact meat samples using phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR); the more sensitive and more rapid non-destructive technique compared with other biochemical analytical methods. The speculated outcome is that ATP level in PSE turkey is greater than that of normal samples at early postmortem stage, as it is continuously generated via glucose oxidation, and rapidly declines afterward. However, there are some difficulties to be recognized, including how quickly meat

samples must be collected after slaughtering, and what optimized conditions to operate ^{31}P-NMR.

It must be noted that PDK4 received priority for an investigation at the protein level in this dissertation. This is due to its substantial down-regulation reported by both transcriptome analyses as well as its biological relevance to development of PSE meat. Other candidate genes can be selected from the other associated differentially expressed genes using similar criteria to confirm the different expression at protein level and explore its biological manifestation in development of the turkey meat defect.

Collectively, we are one step closer to obtaining a complete understanding of molecular mechanisms underlying development of PSE turkey. Pathway intervention approaches can be developed and implemented to minimize occurrence of PSE turkey. For example, based on the findings in this dissertation, an induced expression of *PDK4* is in response of low blood glucose level and glucocorticoids secretion. The use of nutrient modification by reducing percentage of carbohydrate and increasing protein in feed may induce expression of PDK4 and compensate for the impairment of glucose oxidation. Further tests in the turkey cell lines as well as animal subjects are encouraged before an actual application with the population to ensure that the approaches are effective and less likely to generate other problems for the industry.

It is also recommended to determine transcriptome profiles of normal and PSE turkey subjected to thermal stress. An increased occurrence of “light meat”

with inferior quality characteristics has been reported when turkeys are exposed to heat stress. As the global average temperature has been rising, weather anomalies, including heat waves and harsh cold temperatures will occur more frequently. Exposure to weather extremes can induce thermal stress in the animals. The fast-growing turkeys display less environmental heat tolerance; thus, the modern commercial birds may struggle with the thermal challenge due to the climate changes and produce more PSE meat. Conducting a series of studies similar to the current study will provide mechanistic comprehension of the PSE problem in the heat stress exposed turkeys. The biochemical information will be beneficial for the development of PSE-intervention approaches that work effectively in both heat-stress and non-heat stress populations.

APPENDICES

APPENDIX A

CLASSIFICATION OF PALE, SOFT AND EXUDATIVE TURKEY MEAT

The problem of PSE meat in the poultry industry has been one of the greatest challenges to both producers and processors. Meat processors contend with undesirable characteristics of processed products made from the PSE meat due to denaturation of myofibrillar proteins. Separation of PSE meat from normal meat before the defective meat enters the process is the most effective strategy to minimize defective products from PSE meat in commercial plants (reviewed by Barbut et al., 2008). However, establishment of sorting criteria precisely representing protein denaturation is still problematic for both industry and scientific investigators.

Previous studies proposed to utilize meat color as a sorting indicator for PSE poultry meat (Barbut 1998) as it can be measured easily and rapidly with a non-destructive method in the processing line. In the poultry industry, color of the meat was originally determined by a visual scoring system, and this method has continued in some plants (Barbut 2002). However, this method is effective only when identifying PSE meat with an extreme discoloration. Poultry PSE is difficult to differentiate from normal meat as the pale appearance of poultry PSE meat is not as visually obvious as in porcine PSE meat due to the differences in muscle structure and composition between species (Aberle et al., 2001).

Measurement of meat color using colorimetric devices has been the most popular method used by scientific investigators and also implemented in some

industrial plants. Colorimetric equipment provides more accurate readings and overcomes the inherent human-error of visual scoring (Barbut, 2002). In the color system, lightness correlates with an increasing L^* value (range from 0 to 100); therefore, the greater the L^* value, the paler the meat is. Development of L^* cutoff appears to be a promising indicator in classifying poultry PSE meat for both industry and scientific study.

However, there is inconsistency among previous reports as to what L^* cutoff should be exploited (Table A.1). Previous investigators also observed that lightness of meat as reported by L^* considerably varies in accordance with many factors, including transportation (Bianchi et al., 2005), and color measuring practices (Barbut, 1997a; Bianchi et al., 2002; Garcia et al., 2010; Petracci and Fletcher, 2002; Wilkins et al., 2000), as well as seasons (Barbut, 1997b; Bianchi et al., 2007; McCurdy et al., 1996; Petracci et al., 2004). Color variation is also found among birds from different genetic lines (Janisch et al., 2011), genders (Chiang et al., 2008) and flocks (Barbut, 1998). Based on previous research, it is difficult to define a stringent L^* cutoff point for classifying poultry PSE meat.

Table A.1 Cutoff values used in previous literature for classifying poultry PSE meat

Investigators	Published year	Sample	Cutoff value	
			PSE	Normal
Barbut	1996	Turkey	$L^* > 50$	(N/A)
McKee and Sams	1997	Turkey	$L^* > 53$	(N/A)
Pietrzak et al.	1997	Turkey	$pH_{20\ min} \leq 5.8$	$pH_{20\ min} > 5.8$
Barbut	1998	Turkey	$L^* \geq 53$	(N/A)
		Chicken	$L^* \geq 50$	(N/A)
Owens et al.	2000	Turkey	$L^* \geq 53$	$L^* < 53$
Petracci et al.	2004	Chicken	$L^* > 56$	$50 \leq L^* \leq 56$
Barbut et al.	2005	Chicken	$L^* > 53$ $pH < 5.7$	$46 < L^* \leq 53$ $5.7 < pH < 6.1$
Bianchi et al.	2005	Chicken	$L^* > 53$	$46 < L^* \leq 53$
Zhang and Barbut	2005	Chicken	$L^* > 53$ $pH < 5.7$	$46 < L^* < 53$ $5.9 < pH < 6.1$
Fraqueza et al.	2006	Turkey	$L^* \geq 50$ $pH_{24\ h} < 5.8$	$40 < L^* < 50$
Marchi et al.	2009	Chicken	$L^* \geq 53$ $pH < 5.9$	$44 \leq L^* \leq 53$ $pH \geq 5.9$
Oda et al.	2009	Chicken	$L^* > 53$	(N/A)
Garcia et al.	2010	Chicken	$L^* \geq 49$	(N/A)
Gorsuch and Alvarado	2010	Chicken	$L^* > 53$	$L^* < 53$
Wilhelm et al.	2010	Chicken	$pH_{1.5\ h} < 6.0$	$pH_{1.5\ h} > 6.0$
Ziober et al.	2010	Chicken	$L^* > 53$	$44 < L^* < 53$
Droval et al.	2012	Chicken	$pH_{1.5\ h} < 6.0$	$pH_{1.5\ h} > 6.0$
Samuel et al.	2012	Chicken	$L^* > 60$	(N/A)
Sporer et al.	2012	Turkey	Low %marinade uptake High %cook loss	High %marinade uptake Low %cook loss
Zhu et al.	2012	Chicken	$L^* > 53$	$48 < L^* \leq 53$

Our group also experienced variation of meat lightness among turkey samples from different genetic lines (Figure A.1). The turkey breasts of randombred control (**RBC2**) line birds were darker than those of the modern commercial (**COMM**) line. If the samples were classified as PSE when L^* > 53, the cutoff generally used in most previous research, none of the samples from the COMM line would be considered “normal” samples. In addition, lightness of the meat samples from both lines showed no significant correlation with marinade uptake ($P \geq 0.05$, Figure A.2). For cook loss, weak positive correlation between lightness and cook loss was found in RBC2 turkey meat ($P < 0.05$), while no significant correlation was found in COMM samples ($P \geq 0.05$). High marinade uptake and low cook loss represent superior functionality, particularly water holding capacity, of myofibrillar proteins. The results implied that L^* value may not be an accurate predictor of protein functionality which agrees with the observation of Samuel et al. (2012) reporting a weak correlation between lighter color of poultry meat and water holding capacity.

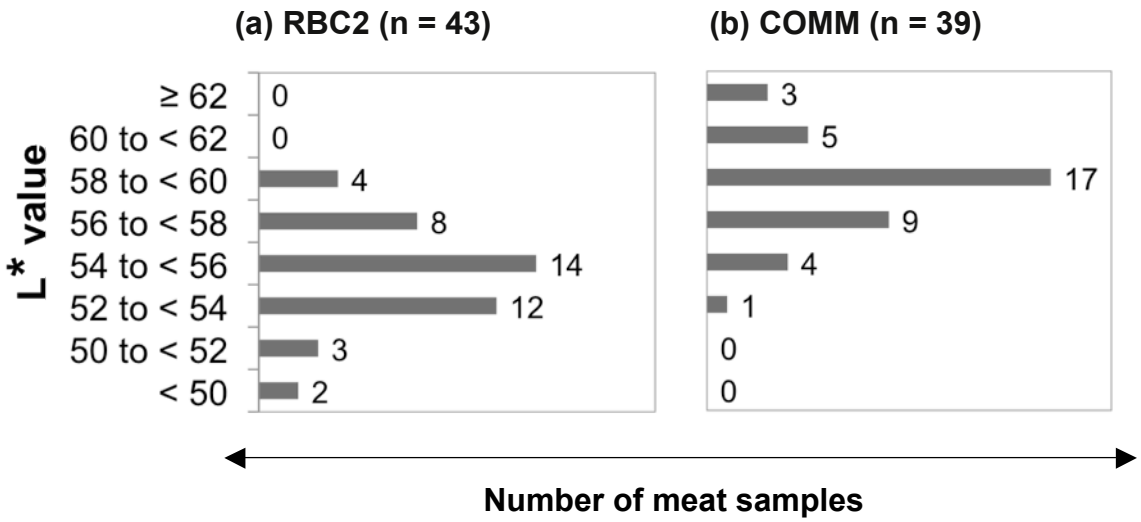


Figure A.1 Color distribution of turkey breast meat. Meat samples were collected from (a) the randombred control line (RBC2, n = 43) or (b) the modern commercial line (COMM, n = 39).

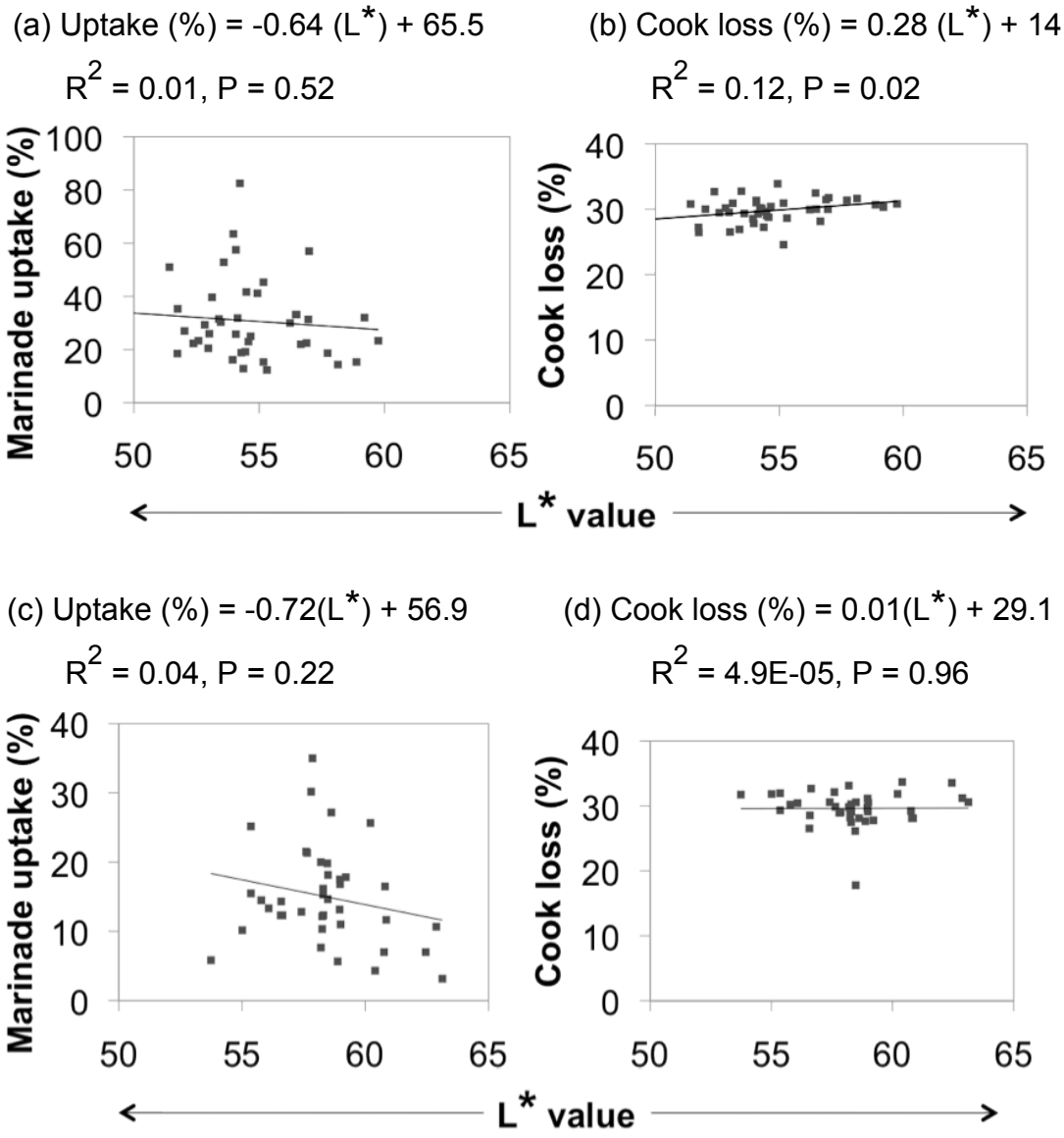


Figure A.2 Correlations between lightness (L^*) and meat quality indices (percent marinade uptake or percent cook loss) of turkey breasts. Samples were collected from randombred control (RBC2) turkeys (n = 43) and modern commercial (COMM) turkeys (n = 39). (a, b) Correlations between L^* and meat quality of RBC2 turkeys. (c, d) Correlations between L^* and meat quality of COMM turkeys.

Based on weak correlations of L^* value and indices for protein functionality, our group decided to use extremes of percentage of marinade uptake and cook loss as primary and secondary indices to classify turkey breast meat as normal or PSE samples within each treatment group (Sporer et al., 2012). The poor water holding capacity of myofibrillar proteins of raw and cooked meat can be indirectly indicated by low marinade uptake and high cook loss. Therefore, the samples with this characteristic would be classified as PSE samples. Conversely, high marinade uptake and low cook loss indicate better protein functionality of myofibrillar proteins; thus, they would be classified as normal samples. We argue that the classification of samples by these criteria provide better representation for this study, which focuses on comparison of gene expression patterns and molecular functions between sample groups with PSE or normal meat quality.

APPENDIX B

DIFFERENTIALLY EXPRESSED TRNASCRIPTS IDENTIFIED BY RNA-SEQ ANALYSIS (FDR<0.05)

Table B.1 Differentially expressed transcript identified in RNA-Seq (false discovery rate; FDR<0.05)

Gene ID	Reference gene name	FC[1]	FDR
AADAC	arylacetamide deacetylase (esterase)	-2.1	9.41E-06
ABAT	4-aminobutyrate aminotransferase	-1.9	2.38E-04
ABCA5	ATP-binding cassette, sub-family A (ABC1), member 5	-2.2	1.41E-04
ABHD5	abhydrolase domain containing 5	-1.9	6.73E-04
ABLIM3	actin binding LIM protein family, member 3	1.8	1.73E-02
ACAA2	acetyl-CoA acyltransferase 2	1.6	1.87E-02
ACACB	acetyl-coenzyme A carboxylase beta	-1.5	4.76E-02
ACBD7	acyl-CoA binding domain containing 7	-2.3	3.40E-03
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	2.0	1.40E-02
ACOT11	acyl-CoA thioesterase 11	-4.0	1.69E-17
ACSBG2	acyl-CoA synthetase bubblegum family member 2	1.7	1.53E-02
ACTA2	actin, alpha 2, smooth muscle, aorta	1.9	6.95E-04
ACTC1	actin, alpha, cardiac muscle 1	7.2	1.29E-33
ACTG2	actin, gamma 2, smooth muscle, enteric	3.0	1.12E-06
ADAMTS 12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	2.3	1.04E-04
ADAMTS 15	ADAM metallopeptidase with thrombospondin type 1 motif, 15	2.5	5.87E-07
ADAMTS 2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	1.9	8.31E-04
ADAMTS 9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	1.8	3.20E-03
ADIPOQ	adiponectin, C1Q and collagen domain containing	1.9	2.41E-04
ADIPOR2	adiponectin receptor 2	-2.0	1.31E-04
ADPRHL 1	ADP-ribosylhydrolase like 1	-1.6	3.02E-02
AEBP1	AE binding protein 1	1.8	6.61E-03

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
AGBL1	ATP/GTP binding protein-like 1	2.5	3.07E-09
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	-1.7	6.63E-03
AGRN	agrin	2.1	5.19E-03
AMD1	adenosylmethionine decarboxylase 1	1.9	3.50E-04
ANGPT4	angiopoietin 4	2.0	3.93E-03
ANGPTL1	angiopoietin-like 1	2.4	4.31E-02
ANKRD24	ankyrin repeat domain 24	-10.4	1.40E-15
ANO6	anoctamin 6	-1.6	2.73E-02
ANXA1	annexin A1	2.1	9.04E-04
ANXA2	annexin A2	1.5	2.83E-02
ANXA7	annexin A7	1.8	1.05E-02
APP	amyloid beta (A4) precursor protein	1.9	1.09E-03
ARL4C	ADP-ribosylation factor-like 4C	3.3	1.45E-02
ARRDC2	arrestin domain containing 2	-6.5	6.71E-34
ASB2	ankyrin repeat and SOCS box containing 2	-2.2	9.40E-06
ASB4	ankyrin repeat and SOCS box containing 4	-1.8	1.19E-03
ASNS	asparagine synthetase (glutamine-hydrolyzing)	2.1	4.88E-04
ASPA	aspartoacylase	-2.7	3.14E-04
ATF3	activating transcription factor 3	1.6	1.57E-02
ATOH8	atonal homolog 8 (Drosophila)	-2.3	4.89E-05
ATP1B4	ATPase, Na+/K+ transporting, beta 4 polypeptide	-5.2	5.48E-25
ATP5I	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit E	1.5	3.62E-02
B1N1B8_MELGA	Uncharacterized protein	-3.7	8.38E-09
B1N1B8_MELGA	Uncharacterized protein	-2.8	1.51E-07
B3VL40_MELGA	delta-like 1 homolog (Drosophila)	-2.0	3.42E-02
BBOX1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1	-1.7	1.32E-02
BCAT1	branched chain amino-acid transaminase 1, cytosolic	2.3	5.18E-06

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
BCHE	butyrylcholinesterase	3.3	6.43E-03
BDH1	3-hydroxybutyrate dehydrogenase, type 1	-1.8	5.19E-03
BEST3	bestrophin 3	-3.2	2.86E-12
BMF	Bcl2 modifying factor	-2.7	1.00E-08
BOC	Boc homolog (mouse)	1.7	3.45E-02
C10orf26	WW domain binding protein 1-like	-2.3	5.07E-07
C13orf39	methyltransferase like 21C	2.2	8.81E-07
C1orf96	chromosome 1 open reading frame 96	-2.3	4.09E-02
C1QTNF1	C1q and tumor necrosis factor related protein 1	3.6	2.34E-03
C1QTNF2	C1q and tumor necrosis factor related protein 2	2.2	3.42E-04
C1S	complement component 1, s subcomponent	1.8	4.18E-02
C2CD3	C2 calcium-dependent domain containing 3	-2.8	1.11E-03
C2orf40	chromosome 2 open reading frame 40	2.6	1.55E-03
CA4	carbonic anhydrase IV	4.0	7.72E-09
CACNG4	calcium channel, voltage-dependent, gamma subunit 4	2.3	1.50E-04
CARNS1	carnosine synthase 1	-1.5	4.09E-02
CAT	catalase	-2.5	2.79E-08
CCDC135	coiled-coil domain containing 135	-1.6	4.19E-02
CCDC141	coiled-coil domain containing 141	-1.9	1.77E-04
CCDC80	coiled-coil domain containing 80	1.8	1.99E-03
CCPG1	cell cycle progression 1	-1.7	2.52E-03
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	-2.3	3.19E-04
CD34	CD34 molecule	1.6	4.04E-02
CD59	CD59 molecule, complement regulatory protein	1.7	2.66E-02
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	1.7	4.19E-02
CENPV	centromere protein V	1.7	1.35E-02
CERCAM	cerebral endothelial cell adhesion molecule	1.7	4.72E-02
CHAD	chondroadherin	2.8	1.49E-08
CHMP6	charged multivesicular body protein 6	-1.5	4.64E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
CHODL	chondrolectin	2.2	2.54E-02
CHRND	cholinergic receptor, nicotinic, delta	3.4	6.99E-13
CIDEC	cell death-inducing DFFA-like effector c	-2.5	4.09E-02
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	-2.2	1.73E-05
CLEC3B	C-type lectin domain family 3, member B	2.0	6.72E-05
CLTA	clathrin, light chain A	1.8	3.58E-02
CLU	clusterin	1.6	1.82E-02
CNR1	cannabinoid receptor 1 (brain)	2.4	5.98E-05
CNTN4	contactin 4	2.2	1.25E-02
COL16A1	collagen, type XVI, alpha 1	2.2	1.31E-03
COL1A2	collagen, type I, alpha 2	3.9	7.89E-17
COL20A1	collagen, type XX, alpha 1	3.9	2.49E-05
COL21A1	collagen, type XXI, alpha 1	2.5	5.16E-03
COL28A1	collagen, type XXVIII, alpha 1	1.7	1.06E-02
COL3A1	collagen, type III, alpha 1	3.3	2.35E-08
COL4A6	collagen, type IV, alpha 6	2.0	3.88E-02
COL5A1	collagen, type V, alpha 1	2.7	1.26E-09
COL5A2	collagen, type V, alpha 2	2.0	1.44E-04
COL6A1	collagen, type VI, alpha 1	2.8	1.92E-08
COL6A2	collagen, type VI, alpha 2	2.6	7.66E-09
COL6A3	collagen, type VI, alpha 3	1.7	8.96E-03
COQ10B	coenzyme Q10 homolog B (S. cerevisiae)	-1.7	1.00E-02
CPE	carboxypeptidase E	2.7	3.54E-03
CPEB4	cytoplasmic polyadenylation element binding protein 4	-1.6	3.09E-02
CREB3L1	cAMP responsive element binding protein 3-like 1	1.7	2.18E-02
CREB5	cAMP responsive element binding protein 5	2.0	8.71E-06
CRYAB	crystallin, alpha B	-2.0	5.35E-05
CUBN	cubilin (intrinsic factor-cobalamin receptor)	1.8	2.83E-02
CXCL12	chemokine (C-X-C motif) ligand 12	2.0	1.90E-04
CXCL14	chemokine (C-X-C motif) ligand 14	2.0	3.19E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
DACT1	dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)	1.8	2.83E-02
DBX1	developing brain homeobox 1	2.3	3.03E-03
DCBLD1	discoidin, CUB and LCCL domain containing 1	2.0	1.37E-03
DCN	decorin	1.9	3.61E-04
DCTN3	dynactin 3 (p22)	1.6	1.60E-02
DDB2	damage-specific DNA binding protein 2, 48kDa	1.7	2.52E-02
DDR2	discoidin domain receptor tyrosine kinase 2	2.5	7.24E-04
DEGS1	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)	1.8	8.52E-04
DENND2C	DENN/MADD domain containing 2C	-1.7	7.65E-04
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	-1.9	2.54E-02
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	-1.7	1.61E-02
DNASE1L3	deoxyribonuclease I-like 3	-2.2	3.31E-04
DUSP26	dual specificity phosphatase 26 (putative)	-2.0	3.25E-03
DUSP8	dual specificity phosphatase 8	-1.8	5.82E-04
ECM2	extracellular matrix protein 2, female organ and adipocyte specific	1.8	7.90E-03
EDA2R	ectodysplasin A2 receptor	2.1	1.25E-05
EGF	epidermal growth factor	1.6	1.01E-02
EGR1	early growth response 1	1.8	7.90E-03
EHHADH	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	-2.2	3.63E-02
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	1.8	1.98E-02
ELN	elastin	14.3	1.20E-55
EMILIN2	elastin microfibril interfacer 2	1.7	7.11E-03
ENAH	enabled homolog (Drosophila)	1.6	3.77E-03
EPHB1	EPH receptor B1	1.7	6.68E-03
EPN3	epsin 3	-2.5	5.64E-06
ESR1	estrogen receptor 1	-2.1	8.06E-04
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.6	3.37E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
FAM117A	family with sequence similarity 117, member A	-1.7	3.94E-03
FAM13A	family with sequence similarity 13, member A	-3.0	1.41E-12
FAM173B	family with sequence similarity 173, member B	-2.1	4.98E-03
FAM46A	family with sequence similarity 46, member A	1.8	9.38E-03
FAM55C	family with sequence similarity 55, member C	-2.3	2.66E-06
FAM70A	family with sequence similarity 70, member A	-2.1	5.28E-05
FBLN1	fibulin 1	1.9	3.25E-03
FBLN2	fibulin 2	1.7	8.60E-03
FBP2	fructose-1,6-bisphosphatase 2	1.6	1.55E-02
FBXO32	F-box protein 32	-6.5	5.10E-32
FGFR1	fibroblase growth factor receptor 1	1.6	4.70E-02
FHL1	four and a half LIM domains 1	1.9	5.92E-05
FHL2	four and a half LIM domains 2	2.7	1.92E-05
FHOD3	orming homology 2 domain containing 3	1.8	8.41E-04
FKBP10	FK506 binding protein 10, 65 kDa	2.5	3.64E-06
FKBP5	FK506 binding protein 5	-2.5	2.59E-08
FKTN	fukutin	2.9	3.86E-12
FMOD	fibromodulin	5.4	2.03E-15
FN1	fibronectin 1	1.9	1.07E-03
FNDC1	fibronectin type III domain containing 1	2.8	9.72E-09
FNDC5	fibronectin type III domain containing 5	2.2	3.39E-03
FSCN1	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	2.3	9.20E-04
FSTL1	follistatin-like 1	2.1	3.69E-05
FYN	FYN oncogene related to SRC, FGR, YES	2.0	5.96E-04
G0S2	G0/G1 Switch protein 2	1.7	4.90E-02
GABRA4	gamma-aminobutyric acid (GABA) A receptor, alpha 4	1.9	3.50E-02
GADD45A	growth arrest and DNA-damage-inducible, alpha	-1.5	4.10E-02
GADL1	glutamate decarboxylase-like 1	1.6	2.58E-02
GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	3.5	6.44E-04

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
GALNTL1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1	2.8	1.33E-04
GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	2.6	6.81E-09
GDAP1	ganglioside-induced differentiation-associated protein 1	-2.0	1.75E-02
GDF8_MELGA	Growth/differentiation factor 8	2.1	5.76E-05
GGT5	gamma-glutamyltransferase 5	2.1	1.41E-02
GJA1	gap junction protein, alpha 1, 43kDa	2.0	2.20E-04
GLRX	glutaredoxin (thioltransferase)	1.7	3.76E-03
GLUL	glutamate-ammonia ligase	-2.5	1.36E-09
GPRC5C	G protein-coupled receptor, family C, group 5, member C	-2.5	6.10E-04
GRB10	growth factor receptor-bound protein 10	-2.2	3.46E-05
GRK7	G protein-coupled receptor kinase 7	4.1	8.02E-06
GTSF1	gametocyte specific factor 1	-3.3	2.95E-06
GYLTL1B	glycosyltransferase-like 1B	1.6	3.16E-02
HAPLN3	hyaluronan and proteoglycan link protein 3	2.1	7.94E-03
HBA_MELGA	Uncharacterized protein	1.8	2.21E-03
HBAD_MELGA	Uncharacterized protein	2.8	1.64E-06
HDAC9	histone deacetylase 9	1.7	8.19E-03
HECTD2	HECT domain containing 2	-2.3	4.01E-04
HIC1	hypermethylated in cancer 1	1.6	2.70E-02
HTRA3	hypermethylated in cancer 1	1.9	4.16E-03
IGFBP3	insulin-like growth factor binding protein 3	1.6	4.11E-02
IGFBP4	insulin-like growth factor binding protein 4	1.8	7.85E-03
IGFBP7	insulin-like growth factor binding protein 7	1.7	9.04E-03
IGFN1	immunoglobulin-like and fibronectin type III domain containing 1	-1.9	3.17E-02
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	4.0	6.50E-08
IGSF3	immunoglobulin superfamily, member 3	1.6	2.06E-02
IL13RA2	interleukin 13 receptor, alpha 2	5.1	4.16E-03

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
INSIG1	insulin induced gene 1	1.8	2.29E-03
IRF8	interferon regulatory factor 8	-1.6	3.85E-02
ISM1	isthmin 1 homolog (zebrafish)	4.8	1.84E-04
ISM2	isthmin 2 homolog (zebrafish)	1.9	7.01E-04
ITGA11	integrin alpha 11	1.8	4.70E-02
ITGA6	integrin, alpha 6	2.1	7.53E-05
ITM2C	integral membrane protein 2C	-2.1	3.06E-05
JAM3	junctional adhesion molecule 3	2.5	3.35E-06
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	3.2	4.69E-06
KBTBD5	kelch repeat and BTB (POZ) domain containing 5	1.8	2.17E-03
KCNC1	potassium voltage-gated channel, Shaw-related subfamily, member 1	2.4	3.28E-04
KCNIP2	Kv channel interacting protein 2	2.8	8.38E-06
KCTD20	potassium channel tetramerisation domain containing 20	-1.7	7.19E-03
KIAA0408	KIAA0408	-2.6	1.50E-07
KIRREL	kin of IRRE like (Drosophila)	1.6	1.92E-02
KLF15	Kruppel-like factor 15	-2.2	3.99E-06
KLHL23	Uncharacterized protein	-1.9	1.25E-02
KLHL24	Uncharacterized protein	-2.6	4.22E-09
KLHL38	Uncharacterized protein	-21.5	2.54E-59
KY	kyphoscoliosis peptidase	1.6	3.44E-02
LAMP2	lysosomal-associated membrane protein 2	-1.7	2.60E-03
LEPREL4	leprecan-like 4	2.3	1.27E-02
LGALS1	lectin, galactoside-binding, soluble, 1	2.2	2.24E-04
LGI2	leucine-rich repeat LGI family, member 2	1.7	2.73E-02
LIMA1	LIM domain and actin binding 1	1.7	4.32E-02
LINGO1	leucine rich repeat and Ig domain containing 1	-1.7	1.25E-02
LIPA	lipase A, lysosomal acid, cholesterol esterase	1.7	4.39E-02
LMO4	LIM domain only 4	1.6	3.77E-02
LRFN2	leucine rich repeat and fibronectin type III domain containing 2	2.8	1.11E-03
LRRC17	leucine rich repeat containing 17	2.0	3.94E-03
LRRC8A	leucine rich repeat containing 8 family, member A	1.7	7.53E-03

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
LTBP1	latent transforming growth factor beta binding protein 1	1.8	4.47E-03
LTBP2	latent transforming growth factor beta binding protein 2	1.7	4.59E-03
LUM	lumican	2.3	1.03E-06
MAB21L1	mab-21-like 1 (C. elegans)	3.6	3.64E-03
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	-1.8	3.75E-02
MAMDC2	MAM domain containing 2	2.5	6.33E-06
MAP1B	microtubule-associated protein 1B	-2.6	1.26E-05
MAP2K6	mitogen-activated protein kinase kinase 6	-1.7	7.98E-03
MAP3K15	mitogen-activated protein kinase kinase kinase 15	-1.8	5.07E-03
MAP3K4	mitogen-activated protein kinase kinase kinase 4	1.7	2.80E-04
MAPRE2	microtubule-associated protein, RP/EB family, member 2	-2.0	1.93E-04
MBOAT2	membrane bound O-acyltransferase domain containing 2	2.2	4.99E-03
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	-1.8	6.06E-03
MFAP5	microfibrillar associated protein 5	1.7	1.58E-02
MLF1	myeloid leukemia factor 1	1.5	3.59E-02
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	Inf	1.79E-03
MRC2	mannose receptor, C type 2	1.7	3.30E-03
MRPL50	mitochondrial ribosomal protein L50	1.6	3.52E-02
MTAP	methylthioadenosine phosphorylase	1.6	3.80E-02
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	2.2	3.92E-06
MTMR3	myotubularin related protein 3	-12.4	4.58E-29
MXI1	MAX interactor 1	-2.1	2.38E-05
MXRA8	matrix-remodelling associated 8	1.9	2.19E-03
MYBPC1	myosin binding protein C, slow type	1.9	4.76E-04
MYH10	myosin, heavy chain 10, non-muscle	1.5	4.19E-02
MYH11	myosin, heavy chain 11, smooth muscle	2.3	3.27E-07
MYH13	myosin, heavy chain 13, skeletal muscle	-4.9	1.01E-17
MYL2	myosin, light chain 2, regulatory, cardiac, slow	1.6	1.26E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
MYL9	myosin, light chain 9, regulatory	2.0	1.11E-03
MYLK_MELGA	Uncharacterized protein	1.8	6.61E-03
MYLK4	myosin light chain kinase family, member 4	-4.4	1.99E-20
MYO9A	myosin IXA	1.6	4.49E-02
NDRG4	NDRG family member 4	1.8	2.18E-02
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	3.2	1.48E-09
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	-1.6	1.83E-02
NOTUM	notum pectinacetylesterase homolog	1.7	2.69E-02
NOV	nephroblastoma overexpressed gene	37.7	1.65E-64
NRBP1	nuclear receptor binding protein 1	1.6	3.63E-02
NRP1	neuropilin 1	1.7	1.41E-02
NRXN1	neurexin 1	1.6	3.19E-02
NTN4	netrin 4	-1.9	3.12E-02
NTSR1	neurotensin receptor 1 (high affinity)	1.9	1.04E-03
NUAK1	NUAK family, SNF1-like kinase, 1	-1.7	2.63E-02
OAF	OAF homolog (Drosophila)	2.2	1.46E-04
OGN	osteoglycin	2.5	4.65E-07
OIT3	oncoprotein induced transcript 3	2.3	6.55E-03
OLFML2B	olfactomedin-like 2B	1.7	7.05E-03
OSR1	odd-skipped related 1 (Drosophila)	2.5	1.23E-02
P84479_MELGA	Uncharacterized protein	1.8	3.88E-03
PAOX	polyamine oxidase (exo-N4-amino)	-2.1	9.06E-03
PAQR7	progestin and adipoQ receptor family member VII	-3.3	3.62E-06
PARP3	poly (ADP-ribose) polymerase family, member 3	-2.0	4.05E-04
PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	-1.8	1.21E-03
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	-2.0	2.33E-04
PDE10A	phosphodiesterase 10A	-5.8	1.39E-19
PDGFRB	platelet-derived growth factor receptor, beta polypeptide	1.9	6.73E-04

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
PDGFRL	platelet-derived growth factor receptor-like	2.4	1.23E-04
PDK4	pyruvate dehydrogenase kinase, isozyme 4	-14.1	9.84E-56
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	-4.2	1.95E-16
PHACTR2	phosphatase and actin regulator 2	1.8	3.92E-03
PHYHD1	phytanoyl-CoA dioxygenase domain containing 1	2.2	7.80E-06
PIK3IP1	phosphoinositide-3-kinase interacting protein 1	-1.7	3.30E-03
PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	-4.2	2.11E-02
PLA2G15	phospholipase A2, group XV	-1.7	5.38E-03
PLCD4	phospholipase C, delta 4	-1.6	3.26E-02
PLEKHN1	pleckstrin homology domain containing, family N member 1	1.7	1.82E-02
PLIN1	perilipin 1	-1.7	1.53E-02
PLK2	polo-like kinase 2	2.1	3.09E-03
PLXND1	plexin-D1	1.5	4.84E-02
PM20D2	peptidase M20 domain containing 2	-1.6	2.38E-02
PMM1	phosphomannomutase 1	-1.9	1.59E-03
POSTN	periostin, osteoblast specific factor	4.1	9.76E-18
PPAP2B	phosphatidic acid phosphatase type 2B	1.6	2.26E-02
PPARD	peroxisome proliferator-activated receptor delta	-2.0	4.07E-03
PPIC	peptidylprolyl isomerase C (cyclophilin C)	3.3	1.52E-08
PPP2R2C	protein phosphatase 2, regulatory subunit B, gamma	-2.3	5.38E-03
PPTC7	protein phosphatase 2C homolog 7	-1.6	4.64E-02
PPYR1	pancreatic polypeptide receptor 1	2.5	2.86E-02
PRKG1	protein kinase, cGMP-dependent, type I	1.8	3.54E-03
PRODH	proline dehydrogenase (oxidase) 1	2.9	5.39E-10
PTPLA	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	1.7	7.56E-03
PTPRU	protein tyrosine phosphatase, receptor type, U	2.3	1.53E-04
PUS1	pseudouridylate synthase 1	-1.6	2.21E-02
PXDNL	peroxidasin homolog (Drosophila)-like	3.8	1.26E-04
Q2PP39_MELGA	Uncharacterized protein	1.5	3.98E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
Q2QF78_ MELGA	Uncharacterized protein	-3.7	4.72E-10
Q90VX1_ MELGA	ATP synthase subunit alpha	-1.8	6.72E-04
Q90X50_ MELGA	Uncharacterized protein	-1.9	1.52E-03
RAB3IP	RAB3A interacting protein (rabin3)	-2.2	2.54E-02
RASGEF 1B	RasGEF domain family, member 1B	2.7	4.66E-03
RBM20	RNA binding motif protein 20	-1.9	4.59E-04
REEP5	receptor accessory protein 5	1.8	3.19E-03
RELL1	RELT-like 1	1.6	2.51E-02
RERGL	RERG/RAS-like	1.8	3.52E-02
RGS2	regulator of G-protein signaling 2, 24kDa	-3.4	2.13E-16
RGS7BP	regulator of G-protein signaling 7 binding protein	-3.0	3.44E-02
RNF20	ring finger protein 20	1.5	3.84E-02
RPH3AL	rabphilin 3A-like (without C2 domains)	2.1	1.89E-02
RPIA	ribose 5-phosphate isomerase A	1.5	4.10E-02
RPS23	ribosomal protein S23	1.5	4.86E-02
RREB1	ras responsive element binding protein 1	-2.2	6.91E-06
RRM2B	ribonucleotide reductase M2 B (TP53 inducible)	-1.7	1.12E-02
RTN4RL1	reticulon 4 receptor-like 1	6.8	1.95E-02
S100A10	S100 calcium binding protein A10	1.5	4.10E-02
S100A6	S100 calcium binding protein A6	1.7	4.39E-02
S1PR3	sphingosine-1-phosphate receptor 3	2.3	4.45E-02
SAMD11	sterile alpha motif domain containing 11	-1.6	2.57E-02
SBK2	SH3-binding domain kinase family, member 2	-2.7	2.68E-08
SCG5	secretogranin V (7B2 protein)	2.4	3.29E-03
SEC24D	SEC24 family, member D (S. cerevisiae)	1.8	8.53E-03
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3C	1.5	4.03E-02
SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3D	1.9	5.14E-03
SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3G	-1.8	1.50E-03

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
SEMA6D	sema domain, transmembrane domain ™, and cytoplasmic domain, (emaphoring) 6D	1.7	1.00E-02
SERINC5	serine incorporator 5	1.7	3.25E-02
SERPINE 2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	2.4	1.92E-05
SERPINF 1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	1.7	7.19E-03
SERPINF 2	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	2.8	1.89E-05
SERPINH 1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	1.9	3.22E-04
SESN1	sestrin 1	-4.0	9.29E-20
SESN3	sestrin 3	2.0	1.39E-03
SFRP2	secreted frizzled-related protein 2	3.3	4.75E-07
SGSM2	small G protein signaling modulator 2	-2.2	1.28E-04
SIK2	salt-inducible kinase 2	-1.6	1.78E-02
SKA1	spindle and kinetochore associated complex subunit 1	-2.5	3.31E-02
SKIV2L2	superkiller viralicidic activity 2-like 2 (S. cerevisiae)	1.6	2.54E-02
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	-2.6	8.38E-06
SLC25A2 4	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24	1.6	1.91E-02
SLC25A2 9	solute carrier family 38, member 2	-3.1	2.79E-08
SLC38A2	solute carrier family 43, member 2	1.6	2.73E-02
SLC43A2	solute carrier family 43, member 2	-5.2	1.46E-27
SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	3.3	8.14E-03
SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system), member 5	2.5	1.17E-07
SLIT3	slit homolog 3 (Drosophila)	4.7	1.74E-06
SMPX	small muscle protein, x-linked	1.5	4.52E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	1.8	8.04E-03
SPON2	spondin 2, extracellular matrix protein	1.9	2.67E-03
SPP1	secreted phosphoprotein 1	2.9	4.54E-03
SPSB1	splA/ryanodine receptor domain and SOCS box containing 1	-1.6	1.11E-02
SQSTM1	sequestosome 1	-1.7	1.00E-02
SRPK3	SRSF protein kinase 3	2.2	5.10E-04
SRPX	sushi-repeat containing protein, X-linked	2.1	1.39E-03
ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	-1.8	1.14E-03
STMN1	stathmin 1	1.9	2.46E-02
SYBU	syntabulin (syntaxin-interacting)	3.3	2.30E-12
SYNGR3	synaptogyrin 3	2.2	5.13E-03
SYNM	synemin, intermediate filament protein	1.6	2.40E-02
TAGLN	transgelin	2.9	3.85E-09
TARS	threonyl-tRNA synthetase	1.8	1.19E-03
TBC1D2	TBC1 domain family, member 2	1.7	3.85E-02
TC2N	tandem C2 domains, nuclear	-2.1	2.54E-02
TFPI2	tissue factor pathway inhibitor 2	1.9	3.16E-02
TGFB3	transforming growth factor, beta 3	1.6	6.63E-03
THRSP	thyroid hormone responsive	-1.6	4.14E-02
THY1	Thy-1 cell surface antigen	3.2	3.57E-12
TIMP2	TIMP metallopeptidase inhibitor 2	1.6	2.45E-02
TLN1	talin 1	1.5	3.73E-02
TMED3	transmembrane emp24 protein transport domain containing 3	2.4	2.20E-04
TMEM108	transmembrane protein 108	1.9	7.83E-03
TMEM163	transmembrane protein 163	1.7	3.55E-02
TMEM200A	transmembrane protein 200A	1.8	2.57E-02
TMEM45A	transmembrane protein 45A	2.3	3.93E-03
TMEM9	transmembrane protein 9	-2.4	3.22E-04

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
TMTC1	transmembrane and tetratricopeptide repeat containing 1	-2.0	1.25E-03
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	-1.7	1.53E-02
TRIM63	tripartite motif containing 63	-1.6	3.79E-02
TUBA1C	tubulin, alpha 1c	2.0	6.33E-05
TUBB6	tubulin, beta 6	2.2	3.13E-05
TULP1	tubby like protein 1	-4.1	1.47E-06
TWIST2	twist homolog 2 (Drosophila)	4.2	3.42E-03
UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	-2.3	8.30E-07
UBE2B	ubiquitin-conjugating enzyme E2B	-1.6	1.63E-02
UBXN7	UBX domain protein 7	-1.7	7.05E-03
USP2	ubiquitin specific peptidase 2	-2.3	6.83E-07
USP35	ubiquitin specific peptidase 35	-3.1	4.70E-03
VASH2	vasohibin 2	3.8	2.96E-19
VASN	vasorin	2.2	3.95E-03
VEGFA	vascular endothelial growth factor A	1.8	2.28E-03
VGLL2	vestigial like 2 (Drosophila)	1.7	1.14E-03
VIM	vimentin	1.9	1.89E-03
WDFY1	WD repeat and FYVE domain containing 1	1.9	5.96E-04
WNT16	wingless-type MMTV integration site family, member 16	14.5	4.59E-04
WNT5B	wingless-type MMTV integration site family, member 5B	-1.6	3.16E-02
WNT9A	wingless-type MMTV integration site family, member 9A	-4.9	1.61E-02
XBP1	X-box binding protein 1	2.3	9.97E-07
XPA	xeroderma pigmentosum, complementation group A	2.2	5.24E-06
XPR1	xenotropic and polytropic retrovirus receptor 1	-1.6	1.53E-02
XYLT1	xylosyltransferase I	2.2	6.10E-03
YARS	tyrosyl-tRNA synthetase	1.7	5.73E-03
YIPF7	Yip1 domain family, member 7	-1.6	2.86E-02
ZBTB16	zinc finger and BTB domain containing 16	-6.4	9.35E-32
ZBTB44	zinc finger and BTB domain containing 44	-1.8	4.36E-03

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
ZNF516	zinc finger protein 516	2.5	1.36E-03
unknown	unknown	-569.3	1.62E-55
unknown	unknown	5.5	1.68E-23
unknown	unknown	-8.3	8.02E-20
unknown	unknown	-5.1	2.91E-18
unknown	unknown	3.7	1.79E-11
unknown	unknown	-9.8	2.08E-09
unknown	unknown	-2.7	2.38E-09
unknown	unknown	-2.9	2.06E-08
unknown	unknown	-7.5	3.15E-08
unknown	unknown	-3.5	2.42E-07
unknown	unknown	-4.1	2.46E-07
unknown	unknown	3.4	4.76E-07
unknown	unknown	-2.5	9.67E-07
unknown	unknown	-2.5	1.18E-06
unknown	unknown	2.5	2.17E-06
unknown	unknown	2.5	2.61E-06
unknown	unknown	-2.8	2.99E-06
unknown	unknown	2.6	3.58E-06
unknown	unknown	2.1	7.06E-06
unknown	unknown	-2.2	1.36E-05
unknown	unknown	-6.2	4.32E-05
unknown	unknown	-2.7	4.35E-05
unknown	unknown	2.3	1.03E-04
unknown	unknown	3.3	1.49E-04
unknown	unknown	1.9	1.53E-04
unknown	unknown	-2.0	2.51E-04
unknown	unknown	7.2	3.09E-04
unknown	unknown	4.7	3.09E-04
unknown	unknown	1.9	3.14E-04
unknown	unknown	-2.0	4.88E-04
unknown	unknown	-1.9	5.43E-04
unknown	unknown	1.9	7.29E-04
unknown	unknown	3.5	8.84E-04

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
unknown	unknown	-1.9	1.01E-03
unknown	unknown	-1.8	1.03E-03
unknown	unknown	-12.5	1.44E-03
unknown	unknown	1.8	1.75E-03
unknown	unknown	-1.8	1.98E-03
unknown	unknown	3.6	2.28E-03
unknown	unknown	3.0	2.42E-03
unknown	unknown	2.7	2.93E-03
unknown	unknown	-1.7	3.94E-03
unknown	unknown	5.4	4.44E-03
unknown	unknown	1.7	4.48E-03
unknown	unknown	4.2	5.38E-03
unknown	unknown	-1.7	7.20E-03
unknown	unknown	2.2	7.20E-03
unknown	unknown	-1.7	9.52E-03
unknown	unknown	2.1	9.52E-03
unknown	unknown	-1.8	9.74E-03
unknown	unknown	-1.7	9.86E-03
unknown	unknown	1.6	1.01E-02
unknown	unknown	1.7	1.01E-02
unknown	unknown	1.7	1.20E-02
unknown	unknown	-2.0	1.35E-02
unknown	unknown	-1.8	1.59E-02
unknown	unknown	-1.7	1.71E-02
unknown	unknown	2.6	1.93E-02
unknown	unknown	-1.6	1.99E-02
unknown	unknown	-4.2	2.05E-02
unknown	unknown	1.6	2.13E-02
unknown	unknown	2.4	2.18E-02
unknown	unknown	3.0	2.49E-02
unknown	unknown	1.8	2.51E-02
unknown	unknown	1.7	3.13E-02
unknown	unknown	2.1	3.44E-02
unknown	unknown	-1.5	3.75E-02

Table B.1 (cont'd)

Gene ID	Reference gene name	FC	FDR
unknown	unknown	1.5	3.89E-02
unknown	unknown	1.6	3.95E-02
unknown	unknown	-1.5	4.03E-02
unknown	unknown	2.5	4.03E-02
unknown	unknown	2.2	4.08E-02
unknown	unknown	1.5	4.18E-02
unknown	unknown	2.4	4.25E-02
unknown	unknown	1.6	4.96E-02
unknown	unknown	1.6	4.88E-02

[1] Fold change (FC) indicates ratio of expression of gene in PSE turkey relative to normal samples. Positive FC values show increased expression in PES relative to normal samples; negative FC values indicate decreased expression in PSE sample relative to normal.

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