DIFFERENTIAL GENE EXPRESSION IN LUNG TISSUE OF WOODEN BREAST SYNDROME AFFECTED AND UNAFFECTED COMMERCIAL BROILER CHICKENS

by

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ABSTRACT

For years, the commercial boiler chicken industry has focused its breeding efforts on producing feed-efficient broiler chickens that produce large amounts of meat in a short amount of time to accommodate the high consumer demand for poultry products. The economic welfare of the poultry industry is dependent upon the quality of the meat produced; therefore, the rise in muscle disorders and myopathies that jeopardize meat quality are concerning to growers. This study focuses on the novel muscle disease, Wooden Breast Syndrome, which is characterized by an abnormally hard pectoralis major muscle. Many studies have also identified muscle hypoxia and oxidative stress as significant symptoms of Wooden Breast Syndrome indicative of respiratory system impairments. In particular, this study will characterize gene expression differences in the lung tissues of affected and unaffected commercial broiler chickens using functional analysis tools to determine which specific pathways or cellular functions are affected by this disease. The purpose of this research is to gain a better understanding of the disease etiology of Wooden Breast Syndrome, to understand how it affects the health of commercial broiler chickens, to gain insight into ways to remedy hypoxia and oxidative stress in affected birds, and to understand the lung's contribution to this disease.

Starting in March 2017, a large live animal experiment on Wooden Breast Syndrome was conducted in which approximately 2,100 broilers were raised to market age. After being euthanized by cervical dislocation, birds were examined for Wooden Breast Syndrome, necropsies were performed, and tissue samples were taken from the breast muscle, liver, and lung. For this study, a total of 12 samples were used, including 6 affected (3 females and 3 males) and 6 unaffected (3 females and 3 males).

Differentially expressed genes in the lung tissues between unaffected and affected birds were identified by RNA analysis and functional analysis was completed using IPA® Functional Analysis Software.

A total of 141 differentially expressed genes were identified using Cuffdiff v2.2.1. The top upregulated (heat shock proteins, SPP1, FABP4, etc.) and downregulated (ALB, HSD17B2, SELENOP1, etc.) differentially expressed genes were selected based on the most positive and negative Log_2 fold change, respectively. Functional analysis of the differentially expressed genes using IPA® identified 5 top canonical pathways, several upstream regulators, and diseases or functions that were significantly activated or inhibited in the affected birds. The results suggest Wooden Breast Syndrome affects lipid metabolism and hypoxia and oxidative stress in the lung tissue.

Analysis of the DE genes identified in lung tissue from broilers affected with Wooden Breast Syndrome suggest the lungs do not have a significant role in the development of Wooden Breast Syndrome. There is not enough evidence to support that markers of immune response, hypoxia, and oxidative stress found in affected lung tissues are activated by issues originating from the lung, as these responses can also be attributed to the systemic impact of Wooden Breast Syndrome. A well-supported finding from this research is the altered lipid metabolism in affected lung tissue, which has been consistently observed in various tissues in broilers affected with Wooden Breast Syndrome. These are only preliminary findings; therefore, the lungs' involvement in Wooden Breast Syndrome cannot be entirely dismissed, as additional studies must be completed to explore significant genes and pathways identified by this research.

Chapter 1

BACKGROUND AND LITERATURE REVIEW

As the world population continues to grow, the demand for life-sustaining resources becomes critical. The ability to produce greater amounts of food with fewer resources is a major goal of modern agriculture. For this reason, scientists are exploring methods of improving food cultivation practices to maximize the production and quality of produce and meats.

The poultry industry has experienced decades of improvements in broiler production that have increased the muscle yield and growth rate of chickens. Although economically beneficial, selective breeding of chickens for traits with high marketability may inadvertently induce the development of new muscle disorders. Muscle disorders not only affect the quality of poultry, but also jeopardize the bird's health and welfare. In addition to meat quality being affected by genetics, several research studies argue that product quality can be altered by environmental factors such as how and where the chickens are raised [44, 33, 36].

A novel chicken myopathy, Wood Breast Syndrome, has only recently emerged in the literature. Sihvo et al. published an early characterization of the disease as causing palpably hard breast muscle that becomes chewy and hard when cooked [39]. Wooden Breast Syndrome has emerged as a global issue and only seems to affect commercial broiler chickens, chickens that are bred and raised specifically for meat production. Incidence of these conditions have been reported in many different countries such as Brazil, Italy, Finland, the United States, and the United Kingdom

[42]. As a result of extensive genetic selection and improved nutrition, broiler chickens experience expedited growth and reach much higher weights than ever before, with the majority of that increased weight localized in the breast muscle [41].

Due to increased consumer demand for affordable poultry meat, the broiler industry has grown immensely. According to the Livestock and Poultry: World Markets and Trade Report released in April 2018 by the United States Department of Agriculture, global production is predicted to grow 2% in 2018 to 92.5 million tons and global broiler export projections in 2018 are also 2% higher to a record 11.3 million tons supported by stronger demand from diverse markets, in particular Japan, Cuba, Hong Kong, Angola, Iraq, and Ghana. In the United States of America, production is expected to rise 2% to a record 19 million tons in 2018 because of growth in domestic consumption and exports will rise 3% to approximately 3.2 million tons [28].

The etiology of Wooden Breast Syndrome is still poorly understood. Gene expression studies using breast muscle from affected and unaffected commercial broiler chickens have provided some limited insight into the topic [3]. Using RNA sequencing, the Mutryn et al. was able to identify about 1,500 genes differentially expressed between the two groups out of 11,000 genes with detectable expression in the tissue [8]. The affected breast muscle appeared to possess a unique metabolic signature related to increased oxidative stress, elevated protein levels, muscle degradation, and altered glucose utilization were identified that can be used to accurately classify affected and unaffected commercial chickens and the severity of the disease [1]. Ongoing research is examining differential expression in other tissues,

such as the liver and lung, to gain a more comprehensive understanding of how Wooden Breast Syndrome affects commercial broiler chickens.

Assessing and analyzing genetic data derived from diseased tissues to identify key molecular differences and altered pathways and cellular functions of Wooden Breast Syndrome is crucial to reduce wasted resources and maximize yield in broiler production. Performing this research with sequencing technology, widely available genetic databases, and bioinformatics tools used to analyze and compare lung tissue of commercial broiler chickens with and without Wooden Breast Syndrome contributes to the overall understanding of how this disease manifests in broilers.

1.1 Importance of Meat Quality

The economic welfare of the poultry industry is dependent upon quality, marketable products. Understanding different factors that influence the development of muscle disorders and myopathies in commercial broiler chickens that can jeopardize the quality of the meat produced is an important step in finding a solution to the problem. With a disease of unknown etiology like Wooden Breast Syndrome has damaging effects on poultry meat quality, its global occurrence is extremely concerning to growers and requires immediate research to better comprehend the underlying mechanisms of the disease.

1.1.1 Environmental Factors

Studies show that particular environmental factors, mainly stressors, can affect muscle quality. In a 2002 study, Woelfel et al. identified pre-slaughter handling, stunning methods, and chilling regimens as stressors capable of altering meat quality. These stressors can expedite rigor mortis, the stage where chemical changes in the

muscles cause the limbs of the corpse to stiffen [44]. Accelerating the natural formation of rigor mortis, or the softening process that follows it, can compromise meat tenderness and ultimately lead to protein denaturing [33]. Tenderness, an important element of meat quality, is dependent upon the rate and extent of the chemical and physical changes occurring in the muscle post-mortem. Birds that struggle before or during slaughter deplete the energy in their muscles quicker and rigor mortis develops much faster than normal causing the meat to be tougher and less tender [33].

This pattern is also seen with the other stressors Woelfel et al. identified. Another significant stressor that affects poultry meat quality is the transportation process. If the temperature is too hot or humid during transport, damage to muscle tissue can occur [36]. In addition to physical conditions during transport, it was found that trips lasting three hours or more can result in high stress levels capable of accelerating metabolism, resulting in diminished muscle glycogen content and a high muscle pH. These adverse events contribute to decreased meat quality.

1.1.2 Selective Breeding

Meat quality is affected by new technologies used to cultivate more marketable products. The use of Recombinant DNA (rDNA) in agricultural biotechnology to produce new genetic combinations that enhance certain characteristics of agricultural goods such as better nutrition or increased shelf life, is a reasonably promising technique for improving and increasing the yield of agricultural products. The animal agriculture industry is a direct beneficiary of rDNA technology in the form of genetically modified feed. Genetically modified grains and oilseeds, which tend to be corn and soybeans, respectively, require less fertilizer and fewer pesticides to grow,

while increasing crop yields. In the poultry industry, as well as other animal agriculture, feeding these grains to the animals results in a more sustainable food product and is environmentally-friendly. There has been no scientific evidence of compromised animal health from the ingestion of genetically modified feed ingredients [22].

Selective breeding involves choosing parents with certain characteristics to breed together and produce offspring with more desirable characteristics [43]. In selective breeding, the genetic modification is completed without the explicit addition of any foreign genetic material, i.e., genes to express specific traits that were not present before, into the organism. Commercial broilers are selectively bred for feed efficiency and growth rate, which can result in altered meat composition and may negatively impact various meat quality traits such as tenderness or expedited degradation post-mortem [43].

Broiler chickens are bred to have a much wider and thicker pectoralis major muscle [3]. Genetic selection contributed to an increase in the diameter and length of muscle fibers and according to a 2007 study conducted by Berri et al., as muscle fiber surface area increased, the expected decrease in post-mortem muscle pH and glycolytic potential did not occur, resulting in an increase in pH [16, 3]. Additionally, an increase in the area and density of muscle fibers is known to decrease the capillary supply in broilers. As the primary site for oxygen diffusion in cells, an insufficient capillary network leads to low oxygen supply to the muscle. By selecting for muscularity, the ratio of capillary to myofiber is shifted unfavorably causing an accumulation of metabolic waste in the pectoralis major muscle due to poor gaseous exchange [16]. Such a decrease in oxygen supply can result in premature necrosis or

changes in post-mortem processes potentially leading to poor meat quality. Although selecting for favorable traits in broilers can be economically stimulating, consequences manifest as hypoxia and accumulated metabolic products in the breast muscle, which are key symptoms found in birds affected with Wooden Breast Syndrome.

1.1.3 Economic Impact

The poultry industry is an essential pillar in food production. In the United States alone, more than ninety pounds per capita of chicken were consumed in 2015, making it the number one consumed protein in America [4]. Appropriately, the United States of America has the largest broiler chicken industry in the world [4]. As a multi-billion-dollar industry, producing healthy broiler chickens with high quality meat is a major priority. The goal is to breed birds that produce large amounts of meat in a short amount of time and efficiently convert feed to muscle mass [3].

Farmers are trying to meet the demands and preferences of consumers. Production is shifted towards specific cuts of meat such as breast, thigh, and leg. Other factors like tenderness, texture, juiciness, drip-loss, and shelf-life also contribute to consumer decisions when purchasing poultry [3]. Choices to discontinue purchasing from a particular poultry company by individual consumers at grocery stores, restaurants, or post-processing plants for poor quality products can lead to detrimental economic outcomes for farmers. A disease such as Wooden Breast Syndrome, which renders breast meat hard and is reported to impact up to 50% of individuals in affected flocks, has substantial financial effects on the poultry industry [35]. Many resources invested in raising these chickens are wasted because diseased cuts of breast meat are usually not sold as filets, making them less valuable in the marketplace. Selecting for chickens with high muscle yield and expedited growth also increases the risk for this

production practice to induce muscle myopathies that jeopardize meat quality. As the number of muscle disorders in broiler chickens rise, the poultry industry faces a daunting economic burden [37]. Understanding the genetics and biological mechanisms behind muscle disorders is the first step in improving production in the poultry industry to avoid economic loss.

1.2 Chicken Myopathies

The increased prevalence of muscle disorders among commercial broiler chickens escalates the need for research. Studying underlying mechanisms of how these diseases affect chickens and poultry products can aid in developing improved breeding techniques that will not compromise the poultry industry's economic welfare nor the health of commercial broilers. Nutritional myopathy is found frequently in the poultry industry, as genetic selection for larger and faster growing chickens also increases the bird's susceptibility to oxidative damages and antioxidant deficiency [13]. Characteristics of nutritional myopathy include white striations in the skeletal muscle and lesions predominantly in the pectoral muscle consisting of degeneration of myofibers, with necrosis, mineralization, and concurrent regeneration [13]. Research on nutritional myopathy is often concerned with diet supplementation. Selenium is considered necessary to prevent nutritional myopathy and vitamin E supplementation can significantly decrease the number of damaged fibers in the pectoral muscles [13,8]. Examining nutritional myopathy at the molecular level provides insight on the various biological mechanisms involved in the disease.

1.2.1 Wooden Breast Syndrome

Wooden Breast Syndrome is found in commercial broiler chickens and is characterized by severe hardening of the pectoralis major muscle during growth to market weight [39]. Composition analyses indicate that affected breast meat has higher levels of moisture, fat, collagen, calcium, and sodium, but lower amounts of protein compared to normal breast meat [46]. White striping is a new muscle condition found in chickens and is also often seen in the breast meat of birds affected with Wooden Breast Syndrome. White striping manifests as white fatty lines running in the same direction as the myofibers of the breast muscle and is usually located ventrally where the muscle is stretching more [25]. It is believed that white striping is a result of mineralization of fat on myofibers and general necrosis of the muscle [25]. White striping negatively impacts the economic welfare of the poultry industry. In a 2012 consumer study conducted by Kuttappan et al., it was found that 50% of consumers would not purchase cuts of chicken breast meat with any degree of white striping because it appears fatty and trends show that consumers are more mindful of the amount of fat in the meat they purchase [24].

Genomic, proteomic, and metabolomic studies have shown differences in affected and unaffected breast meat related to muscle hypoxia, oxidative stress, intracellular calcium levels, protein degradation, cellular repair, fibrosis, and glucose utilization [46]. My research focuses on differential gene expression in the lung tissue of Wooden Breast Syndrome affected and unaffected birds. Hypoxia and oxidative stress are symptoms of particular interest because they are indicative of respiratory issues and impaired gaseous exchange. Understanding how Wooden Breast Syndrome affects lungs at the molecular level will hopefully contribute to our overall understanding of the disease.

Chapter 2

ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN LUNG TISSUE OF WOODEN BREAST SYNDROME AFFECTED AND UNAFFECTED COMMERCIAL BROILER CHICKENS

2.1 Introduction

Selective breeding for large breast muscles in commercial broiler chickens does not conjointly increase the efficiency or size of the cardiovascular and respiratory systems. A study conducted by Mahon et al. suggests that by selecting for rapid growth in birds, there is unintentional selection for poor capillary and fascial growth or muscle fiber defects leading to growth-induced myopathies [30]. The rationale for studying the lungs is that insufficient blood supply causes the accumulation of metabolic waste products like carbon dioxide, which results in oxidative stress and tissue damages [29]. Analysis of tissue samples from broilers affected with Wooden Breast Syndrome shows increased exposure to reactive oxygen species, oxidative stress, and inadequate gas exchange, which are all factors affected by lung function [1]. Additionally, affected birds may experience hypoxia, or reduced oxygen supply to tissue, specifically to breast muscle due to lower capillary density in rapidly growing broiler chickens [16]. A deficiency in the amount of oxygen reaching the lungs and breast muscles and decreased gas exchange due to an insufficient cardiovascular system for the size of the bird, together cause an accumulation of metabolic waste in places such as the breast muscle, as well as several other downstream effects.

Therefore, the phenotype observed in Wooden Breast Disease is evidently linked to the functionality of the lungs.

The goal of this research is to characterize gene expression differences between lung tissue of affected and unaffected commercial broiler chickens using functional analysis tools. Evidence suggests that birds affected with wooden breast possess lower capillary density particularly in the pectoralis major, which in turn causes the birds to experience reduced oxygen supply to this muscle [1, 16, 25]. Therefore, by identifying significant genes that are differentially expressed between the lung tissue of Wooden Breast- affected and unaffected chickens, it will be possible to conduct functional analysis to determine which specific pathways or cellular functions to which the genes belong to. This research will be useful in gaining a better understanding of the disease etiology of Wooden Breast, how it affects the health of commercial broiler chickens, and possible solutions to hypoxia in affected birds.

2.2 Methodology

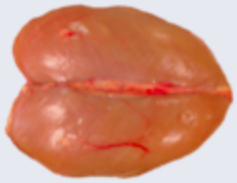
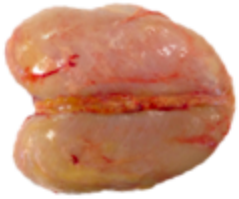
2.2.1 Live Animal Experiment

To investigate the effect of WBD in the transcriptomic profile of the lungs in broiler chickens, a total of 12 samples consisting of affected and unaffected chickens with equal number of males and females were used.

The chicken samples used in this study were selected from a flock of commercial broiler chickens raised from day-old to 53 days post-hatch at the

University of Delaware's poultry houses. These chickens were from a high breast meat yield line of broilers produced by Cobb-Vantress® called Cobb 500. There were three individual hatches of chickens, each containing approximately 700 birds. The second hatch was added four weeks after the first, and the third hatch was added two weeks after the second hatch. All the chickens were raised in conditions mimicking those of commercial settings, with feed and water being provided *ad libitum.* At the end of the experimental period, body weights from the chickens were taken, and then humanely euthanized by cervical dislocation. This was followed by necropsy assessment where the chickens were examined for presence and severity of Wooden Breast Syndrome by manual palpation of the breast muscle. From the final flock of broiler chickens, a total of 6 unaffected and 6 affected chickens comprising 3 males and 3 females in each condition were selected for use in this study, as shown in Table 1. For a distinct separation between unaffected and affected groups, the samples were also selected based on percentage of oxygen saturation in the blood. Broilers with the disease showed significantly lower blood oxygen saturation than unaffected broilers. Samples from all 12 selected chickens were harvested from the cranial region, and snap frozen in liquid nitrogen before storage at -80 ^{o}C until RNA-sequencing.

Table 1. **Study Samples.** A total of twelve lung samples were used in this study, including six from unaffected broiler chickens (top) and six from broilers with Wooden Breast Syndrome (bottom). In each category, three samples were from male birds and three were from female birds. The images in the table are used here to show the drastic visual difference in the pectoralis major muscle between broilers unaffected and affected with Wooden Breast Disease.

SAMPLES	MALE	FEMALE
UNAFFECTED 	3	3
AFFECTED 	3	3

2.2.2 RNA Isolation, Sequencing Protocol, and Analysis

To analyze RNA from the lung samples and investigate differentially expressed genes in the lung tissue of commercial broiler chickens, total RNA was extracted, processed to produce cDNA libraries, and sequenced to generate reads.

All frozen lung samples were initially crushed by hammering with a soft mallet followed by total RNA isolation using mirVana^{TM} miRNA Isolation Kit (Life technologies) following manufacturer's protocol. The concentration of total RNA from all samples was determined using NanoDrop 1000 (Thermo scientific), while

total RNA quality was determined using Fragment analyzer at Delaware biotechnology institute (DBI) at the University of Delaware. Samples with RNA quality number (RQN) of >6 is considered good quality for RNA-sequencing. In this study, the RQN ranged between 7.4 to 10, and were therefore used for RNA-seq. cDNA libraries were constructed using TruSeq Stranded mRNA Sample Prep Kit for low sample (Illumina) using manufacturer's protocol. The concentration and quality of cDNA libraries were checked using Nanodrop and Fragment analyzer respectively prior to sequencing. Nucleotide sequencing was accomplished with Illumina Hiseq 2500 sequencer (DBI) utilizing paired-end sequencing. The resulting raw sequences were then checked for quality using FastQC v0.11.3. All reads passed the quality check and were subsequently mapped to the reference chicken genome (Ensembl Gallus gallus 5.0, (May 2017) and GTF release 89) using Hisat2 v2.1.0 (insert citation) software. Differential gene expression from the lungs between chickens affected with WBD and those without the disease was determined using Cuffdiff software v2.2.1(insert citation). This resulted to generation of a list comprising differentially expressed genes (upregulated and downregulated) in lungs from chickens affected with WBD.

Functional analysis of the differentially expressed (DE) genes was determined using ingenuity pathway analysis (IPA®). To extract significant canonical pathways associated with the significant genes while correcting for multiple tests in IPA®, the Benjamini–Hochberg (B-H) multiple testing correction p-value (<0.05) was used. Besides canonical pathways, other biological functions related to the DE genes in IPA

were explored, such as diseases and functions, upstream regulators as well as regulator effects were examined. Additionally, because the IPA® Functional Analysis software is based off of the human, mouse and rat genome, some of the analyses it produced had to be further scrutinized to align with the chicken genome. For example, a particular gene in a pathway that was identified to have altered functionality based on the uploaded dataset may be present in the human genome, but does not exist in chicken. Therefore, it was important to be mindful of these discrepancies between the reference and chicken genomes when drawing conclusions about the analyses.

2.3 Results

Several observations made during the live animal experiment pertaining to respiration and physical lung characteristics will be noted here for transparency. Although it was not explicitly examined, while raising the three hatches of chickens, respiration did not appear to be labored in any obvious manor. During necropsy, the lung was removed from the bird for tissue collection. Again, the lungs were not closely examined; the goal of this portion of the experiment was to collect tissue samples and identify which birds were unaffected or affected with Wooden Breast Syndrome. Nonetheless, there were no obvious clinical differences between the lungs of affected and unaffected chickens. It is also important to mention that outside of this particular study, histopathological analysis of the same affected and unaffected lung tissue used for this research showed the effects of Wooden Breast Syndrome on lungs are much more prominent in affected males than females.

A total of 141 DE genes were identified using Cuffdiff v2.2.1. Among the top upregulated genes in the lungs of affected chickens included secreted phosphoprotein 1 (SPP1), fatty acid binding protein 4 (FABP4), and several heat shock proteins (HSPB9, LOC772158, HSPA12B), while the most downregulated genes included albumin (ALB), corticotropin releasing hormone binding protein (CRHBP), selenoprotein 1 (SELENOP1), and troponin T2 (TNNT2). The top 10 up-and-down regulated genes are shown in Table 2.

Table 2. **Top 10 Upregulated and Downregulated Genes.** This table is a summary of the top 10 upregulated and downregulated genes found among chickens affected with Wooden Breast Syndrome within the sample population. Positive and negative fold change values reflect, respectively, upregulation and downregulation of gene expression in affected broilers relative to gene expression in unaffected broilers.

Gene Symbols	Gene Name	Unaffected Sample Value	Affected Sample Value	Log_2(Fold Change)
HSPB9	heat shock protein family B (small) member 9	53.2084	860.257	4.01504
GFRA4	GDNF family receptor alpha 4	0.664596	5.87092	3.14304
SPP1	secreted phosphoprotein 1	5.85101	34.6509	2.56614
LOC772158	heat shock protein 30C-like	2.47887	14.2251	2.52068
EEF1A2	eukaryotic translation elongation factor 1 alpha 2	1.28119	6.68462	2.38336
LOC772096	glucagon receptor-like	1.57547	8.0519	2.35355
MMD2	monocyte to macrophage differentiation associated 2	0.301895	1.38191	2.19454
TTR	transthyretin	3.28276	11.7917	1.84479
FABP4	fatty acid binding protein 4	6.57067	23.4481	1.83536
HSPA12B	heat shock 70kD protein 12B	1.56903	5.30222	1.75672
ALB	albumin	14.4786	1.50131	-3.26963

CRHBP	corticotropin releasing hormone binding protein	7.21601	1.40823	-2.35732
TNNT2	troponin T2, cardiac type	3.0325	0.718501	-2.07745
HSD17B2	hydroxysteroid 17-beta dehydrogenase 2	8.92002	2.19415	-2.02338
SELENOP1	selenoprotein P1	132.63	46.6102	-1.50869
B4GALT5	beta-1,4-galactosyltransferase 5	2.01612	0.925869	-1.1227
F10	coagulation factor X	31.3205	15.7419	-0.992491
ZNF217L	zinc finger protein 217-like	6.40066	3.24713	-0.979053
GRIK1	glutamate ionotropic receptor kainate type subunit 1	10.4755	5.31973	-0.977596
ABCC8	ATP binding cassette subfamily C member 8	20.7204	10.9992	-0.913651

2.3.1 Functional Analysis of Differentially Expressed Genes in IPA®

The top canonical pathways, or generalized pathways that represent common properties of a particular signaling module or pathway of specific tissues and cell lines [39], identified by IPA® were LXR/RXR Activation, FXR/ RXR Activation, Acute Phase Response Signaling, Atherosclerosis Signaling, and the Adipogenesis Pathway (Figure 1). LXR/RXR Activation, FXR/ RXR Activation, Atherosclerosis Signaling, and the Adipogenesis Pathway are important in cholesterol (or fat) metabolism. Acute Phase Response Signaling is a key part of inflammatory response.

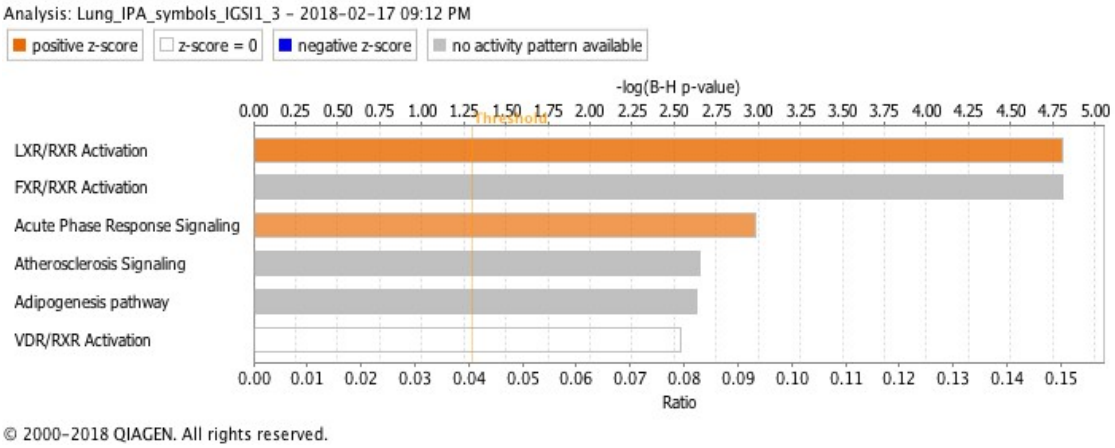


Figure 1. **Top Canonical Pathways.** IPA ® functional analysis software identified these 6 canonical pathways as the most significantly affected based on the DEGs from affected and unaffected broiler lung tissues. Orange indicates a positive z score and gray indicates that within the IPA® program, there is no information on the activity pattern of the pathway. However, that factor does not reject its importance. White indicates a z score of zero, so the VDR/ RXR pathway will not be considered in this study. Additionally, each of these pathways exceed the threshold identified by the vertical orange line. Anything greater than the threshold is significant and anything below is considered not significant.

In Upstream Analysis, upstream regulators are identified and the molecule type and predicted activation state is given among other information related to the gene (Figure 2). These upstream regulators are genes determined by IPA® based on existing literature that, theoretically, are responsible for regulating groups of genes found in the list of 141 DE genes. To narrow the results, a filter was used to only include genes, RNAs, and proteins, which essentially excluded all genes labeled as chemical drugs. The only significantly inhibited upstream regulator is JAG2. IL1, Insulin, Cg, IRS1, TREM1, IL4, CEBPB, FOXO1, IFNG, and TP53 are all significantly activated upstream regulators. JAG2 is a growth factor, IRS1 is an

enzyme, and TREM1 is a transmembrane receptor. IL1, Insulin, and Cg are labeled as group or complex, meaning these are genes that are members of a particular complex. IL4 and IFNG are cytokines. CEBPB, FOXO1 and TP53 are transcription regulators.

Table 3. **Upstream Analysis.** Upstream regulators with significantly different activation states identified by IPA® analysis of DEGs in broiler lung tissue.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score
JAG2	Growth factor	Inhibited	-2.000
IL1	Group	Activated	2.186
Insulin	Group	Activated	2.322
Cg	Complex	Activated	2.434
IRSI	Enzyme	Activated	2.000
TREM1	Transmembrane receptor	Activated	2.213
IL4	Cytokine	Activated	2.220
CEBPβ	Transcription regulator	Activated	2.079
FOXO1	Transcription regulator	Activated	2.200
IFNG	Cytokine	Activated	2.150
TP53	Transcription regulator	Activated	2.478

2.3.2 Diseases and Functions: Downstream Regulators

The Diseases and Functions feature of IPA ® shows downstream regulators and cellular functions that are either decreased or increased based on the DEGs (Figure 3). Several of the Diseases or Functions Annotations are similar and were therefore grouped together for further analysis. For example, migration of phagocytes was increased as well as activation of phagocytes. Intuitively, it makes sense to pair these functions together and display a network of interactions, which is discussed later

in Section 2.4.3. Overall, the downstream analysis identifies alterations in glucose metabolism, fat metabolism, and inflammation.

Table 4. **Diseases and Functions.** IPA® functional analysis software identified the activation of these diseases and functions to be either significantly increased or decreased. Several of them are quite similar and most likely work jointly in either glucose metabolism, fat metabolism, or inflammation.

Diseases or Functions Annotation	Predicted Activation State	Activation Score	Molecules
Survival of organism	Increased	2.851	APOA1, C3, CEBPB, COL18A1, EEF1A2, FZD5, GRIK1, HYAL2, ITGA7, LPL, PSCA, SLN, SPP1, TAP1, THBD, TNNT2
Migration of phagocytes	Increased	2.551	ADIPOQ, ALB, APOA1, C3, CCL19, F10, NPY, SPP1
Metabolism of polysaccharide	Increased	2.412	ADIPOQ, CEBPB, HYAL1, HYAL2, NR4A1, SPP1, THBD
Migration of antigen presenting cells	Increased	2.351	ADIPOQ, APOA1, C3, CCL19, NPY, SPP1
Activation of phagocytes	Increased	2.281	ADIPOQ, APOA1, C3, CEBPB, HSPB8, HYAL2, NPY, SLC6A4, SPP1, TFF2, THY1, TIMD4, TNFRSF6B
Activation of myeloid cells	Increased	2.209	ADIPOQ, C3, CCL26, CEBPB, HYAL2, NPY, SLC6A4, SPP1, TFF2, THY1, TIMD4, TNFRSF6B
Quantity of metal ion	Increased	2.205	ABCC8, ADAMTS1, C3, CCL19, FABP4, GRIK1, NPY, SPP1, THY1, TTR
Metabolism of carbohydrate	Increased	2.163	ABCC8, ADIPOQ, APOA1, CEBPB, ENPP2, FABP4, HYAL1, HYAL2, LCAT, LPL, NPY, NR4A1, SPP1, THBD
Migration of dendritic cells	Increased	2.158	APOA1, C3, CCL19, NPY, SPP1

Activation of leukocytes	Increased	2.031	ADIPOQ, APOA1, BHLHE40, C3, CCL26, CEBPB, CPE, EGR2, F10, HSPB8, HYAL2, NPY, NR4A1, SIGLEC1, SLC6A4, SPP1, TFF2, THY1, TIMD4, TNFRSF6B
Quantity of anion	Increased	2	ADAMTS1, APOA1, HDC, SPP1
Migration of granulocytes	Increased	2	ALB, C3, CCL19, F10, SPP1
Synthesis of reactive oxygen species	Decreased	-2.218	ACP5, ADIPOQ, ALB, APOA1, C3, COL18A1, EEF1A2, HSPB1, LCAT, NPY, NR4A1, SPP1
Conversion of lipid	Decreased	-2.104	ALB, ENPP2, FABP4, HPX, HSD17B2

2.4 Discussion

Due to the vast amount of information provided by IPA ® functional analysis of the DEGs identified from the lung tissues of Wooden Breast Syndrome affected and unaffected birds, this research will focus only on the affected pathways and cellular functions in two major areas: fat metabolism and hypoxia and oxidative stress. Additionally, several upregulated and downregulated genes shown in Table 2 will be analyzed to understand their roles in Wooden Breast Syndrome.

Muscle samples from broilers in the live animal experiment showed high levels of immune response in affected birds, both under the microscope and in DE gene analysis. However, under a microscope, the H&E (hematoxylin and eosin) staining did not show a very large difference in immune response in the lung tissue of broilers affected with Wooden Breast Syndrome compared to unaffected broilers. Although there were some indicators of an increased immune response in affected chickens

based on the list of DE genes, upstream regulators, and canonical pathways, it is not significantly supported by histopathological examination.

2.4.1 Fat Metabolism

A sizable number of DEGs found in the lung tissues of Wooden Breast Syndrome affected and unaffected broilers play a role in fat metabolism. It is known that there are increased levels of fatty acids found in the breast muscle tissue of chickens affected by Wooden Breast Syndrome from the white striping [25]. Interestingly, analysis of the results from lung tissue data shows increased activation of the fat metabolism pathway, suggesting a potential increase in fat content in the lungs as well. Since fat was not seen in the lung tissues during histopathological analysis, the increased fat content in the lungs is most likely ectopic, meaning there is deposition of triglycerides in tissues that normally contain only small amounts of fat, including the lungs [31].

A couple of genes, CCAAT/enhancer binding protein β (CEBPβ) and fatty acid binding protein 4 (FABP4), were highly implicated in the alteration of lipid metabolism in the lungs of broilers affected with Wooden Breast Syndrome.

DE gene analysis and IPA® Upstream Analysis showed that CEBPβ is highly activated in the affected lung tissue (Table 3 and Appendix A). IPA® Canonical Pathway analysis indicated the adipogenesis pathway to be highly activated and notes the Wnt/ β-catenin pathway acts as a molecular switch that governs adipogenesis [18]. A study conducted by Chung et al. showed CEBPβ is involved in the suppression of

Wnt/ β-catenin signaling during adipogenesis by binding directly to the promoter Wnt10b and inhibiting its activity [5]. Activation of Wnt/ β-catenin signaling inhibits adipogenesis, while disruption of Wnt signaling leads to spontaneous adipogenesis. As indicated by the lung DE genes data, CEBPβ is observed to be more activated in affected lung tissue, which could be contributing to suppressed Wnt/ β-catenin signaling caused by decreased Wnt10b promoter activity. Chung et al. noticed the suppression of Wnt signaling during adipogenesis is mainly caused by reduction of Wnt10b expression in mice and tested whether CEBPβ could regulate Wnt10b transcription using differentiation inducers to increase CEBPβ protein levels and cycloheximide to inhibit protein synthesis. They saw that differentiation inducer treatment substantially increased CEBPβ protein level and simultaneously reduced Wnt10b mRNA level in the control cells. Inhibition of protein synthesis by addition of cycloheximide entirely depleted the effect of the differentiation inducer on Wnt10b transcription as well as induction of CEBPβ expression. Even in the presence of cycloheximide, Wnt10b transcription was still repressed in the cells overexpressing CEBPβ. Additionally, Chung et al. observed that knockdown of CEBPβ resulted in slightly recovered Wnt10b mRNA levels at 2 days post-differentiation induction and dramatically recovered Wnt10b mRNA levels at 4 days post-differentiation induction, proposing that CEBPβ does not work alone and may be inducing another factor to inhibit Wnt10b expression. The results from this study [5] suggest that CEBPβ is involved in Wnt10b expression repression, and only directly regulates Wnt10b expression in the early stages of adipogenesis.

Furthermore, CEBPβ stimulates adipogenesis genes such as PPARγ and CEPBα, and Wnt signaling regulates adipogenesis through the inhibition of CEBPα and PPARγ expression [5]. Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors CCAAT/enhancer binding protein α (CEBPα) and peroxisome proliferator- activated receptor γ (PPARγ) [18]. Recently, it has been reported that chicken ovalbumin upstream promoter-transcription factor II, induced by Wnt signaling, acts as a repressor in the PPARγ promoter [34]. Therefore, with highly activated CEBPβ in the affected broiler lung tissue suppressing Wnt signaling, chicken ovalbumin upstream promoter-transcription factor II does not repress PPARγ, which is necessary for fatty acid degradation [45].

Fatty Acid Binding Protein 4 (FABP4) is highly activated in broilers affected with Wooden Breast Syndrome according to DE gene analysis (Table 2). FABP4 is a small cytosolic lipid-binding protein that plays an important role in regulation of lipid homeostasis through its actions in adipocyte fatty-acid uptake, lipogenesis, and delivery of lipids to nuclear receptors mediating nuclear transcriptional programs [6]. FABP4 is one of the most abundant proteins in adipocytes and regulates lipid metabolism by acting on multiple integrated cellular pathways, involving LXR and PPARγ to suppress adipose tissue lipogenesis and promote lipolysis, respectively [11]. Liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR) are two members of nuclear receptors involved in the nutrient metabolisms of dietary fatty acid and cholesterol [45]. In broilers affected with Wooden Breast Syndrome, it is

possible that FABP4 levels are increased as a response to increased adipogenesis induced by the previously mentioned DE genes. Graupera et al. explain that FABP4 regulates lipid metabolism via cellular pathways LXR and PPARγ, which both act in ways to decrease lipid levels. The LXR canonical pathway is activated in affected broilers and PPARγ is also an upregulated DE gene in broilers affected with Wooden Breast Syndrome (Appendix A). An increase in FABP4 levels in lung tissue could also be a response to the increase in fatty acids that subsequently need fatty acid binding proteins to facilitate movement between extra- and intracellular membranes.

2.4.2 Hypoxia and Oxidative Stress

Hypoxia and oxidative stress are symptoms often discussed in studies on Wooden Breast Syndrome. From the live animal experiment alone, a clear distinction in blood oxygen saturation between the affected and unaffected birds was observed. Several DE genes found in the lung tissue, such as heat shock proteins, corticotropin releasing hormone binding protein (CRHBP), selenoprotein P1 (SELENOP1), albumin (ALB), and activated upstream regulator forkhead box protein O1 (FOXO1), appear to play significant roles in the hypoxia and oxidative stress observed in affected broilers.

In this study, the differential expression of several genes within the heat shock family suggest the occurrence of oxidative stress within the lung tissue of affected broiler. A study conducted by Mutryn et al. to characterize Wooden Breast Syndrome observed many DE genes in affected broilers that suggest an increase in ROS in birds affected with Wooden Breast Syndrome [32]. Due to oxidative stress, stress response-related pathways tend to be activated to prevent further damage. These upregulated genes include: heat shock protein family B member 9 (HSPB9), heat shock protein

30C-like (LOC772158), and heat shock protein 70kD protein 12B. Generally, heat shock proteins have many functions, acting as molecular chaperones, guiding protein folding, and preventing protein buildup. In stress-inducing events, such as oxidative stress, the expression of heat shock proteins is known to increase greatly and is used to stimulate a pro-survival response during oxidative damage [20, 7]. Therefore, the upregulation of most heat shock proteins in affected birds indicates the possibility of oxidative stress damages within the lung tissue caused by ROS.

Corticotropin-releasing hormone binding protein (CRHBP) is a downregulated DE gene in broilers affected with Wooden Breast Syndrome. CRHBP binds to corticotropin releasing hormone (CRH), which is a protein important to stress response pathways and has been found to play a role in protecting neurons against oxidative stress [27]. CRHBP may be lowly expressed to allow more free CRH to perform cytoprotective functions against ROS in the lungs of affected broilers.

In this study, selenoprotein P1 (SELENOP1) is significantly downregulated in broilers affected with Wooden Breast Syndrome. Selenoprotein- related deficiencies are associated with oxidative stress damage, as this group of proteins are necessary for counteracting oxidative stress [17]. SELENOP1 is an antioxidant protein and is one of many selenoproteins that serve antioxidant functions against high levels of reactive oxygen species (ROS) [17]. With this protein downregulated, oxidative stress endures due to an imbalance between ROS production and antioxidant defenses.

Albumin (ALB) is the top downregulated gene in the lung tissue of broilers affected with Wooden Breast Syndrome according to DE gene analysis and has a key role in the dynamic surface activity of natural lung surfactant and lipid concentration [2]. Avian surfactant predominantly functions to maintain airflow in tubes and reduces

surface tension, which increases compliance and allows the lung to inflate much more easily. Experiments conducted by Holm et al. characterized the surface property changes from interactions of ALB with natural lung surfactant and extracted lung lipids [2]. They found that ALB in low concentrations had a detrimental effect on the dynamic surface activity of extracted surfactant lipids, consequently impairing gas exchange in the lungs. In addition, ALB also inhibited the isolated adsorption facility of lung surfactant and extracted surfactant lipids. Interestingly, Holm et al. observed the inhibition of surface activity by ALB was moderated or abolished at high lipid concentrations [2]. Even at low concentrations, as seen in DE gene analysis of affected lung tissue, ALB can decrease the efficacy of lung function and gas exchange by impairing dynamic surface tension and adsorption. However, the effects of low ALB concentration in broilers affected with Wooden Breast Syndrome are potentially mitigated by the fact that, as previously described, lipid metabolism in diseased broilers is altered to induce adipogenesis. Perhaps, increased lipid production is a mechanism used by affected chickens in response to hypoxia and impaired lung function to alleviate respiratory stress.

Furthermore, hypoxia, which is often observed in broilers with Wooden Breast Syndrome, induces adipocyte differentiation by triggering reactive oxygen species generation [23]. Oxidative stress induced by generation of excess reactive oxygen species has emerged as a critical, final common mechanism in the atherosclerosis signaling pathway, which is an activated canonical pathway in lung tissue of affected broilers according to IPA® [21]. Atherosclerosis is a chronic inflammatory disease characterized by accumulation of lipids and inflammatory cells in the walls of medium and large-sized arteries [14]. With this, it is clear that hypoxia-inducing adipogenesis

is linked to increased atherosclerosis in the lung tissue of Wooden Breast Syndrome affected broiler chickens. In a cyclic manner, the increased lipid concentration due to hypoxia-induced adipogenesis may be a mechanism to reduce respiratory stress.

Forkhead box protein O1 (FOXO1) is identified by IPA® as an activated upstream regulator in the lung tissue of broilers affected with Wooden Breast Syndrome. Although this protein is not determined to be one of the 141 DE genes identified in diseased broiler lungs using Cuffdiff v2.2.1, it may play a unique role in Wooden Breast Syndrome etiology in the context of pulmonary hypertension (PH) and ascites. The intensive selection of broilers for maximal body mass has resulted in anatomical and physiological limitations of blood flow through the lungs, resulting in insufficient oxygenation of the tissues; consequently, pulmonary hypertension syndrome can develop due to increased pressure in the pulmonary arteries when the heart tries to pump more blood through the lungs to meet the body's oxygen requirement [15, 19]. According to Hargis et al. in the Merck Vet Manual, ascites is an accumulation of non-inflammatory transudate in one or more of the peritoneal cavities or potential spaces resulting from increased vascular hydraulic pressure, vascular damage, increased tissue oncotic pressure, or decreased vascular oncotic pressure, and most commonly, ascites is associated with venous hypertension resulting from right heart failure in response to increased pulmonary resistance [15]. It is well documented that most cases are caused by a genetic predisposition to pulmonary hypertension, and in many situations, this can progress to congestive heart failure and terminal ascites. Commercial broiler chickens affected with Wooden Breast syndrome do not experience ascites; however, blood parameters of diseased birds, such as low oxygen saturation and high CO_2, are very similar to birds experiencing ascites.

A study on the role of FOXO1 in PH showed that in human and experimental PH lungs, FOXO1 was downregulated [46]. They found pharmacological inhibition and genetic ablation of FoxO1 in smooth muscle cells reproduced PH features *in vitro* and *in vivo*. However, pharmacological reconstitution of FOXO1 activity using intravenous or inhaled paclitaxel, or reconstitution of the transcriptional activity of FOXO1 by gene therapy, reversed vascular remodeling and right-heart hypertrophy *in vivo.* In broilers with Wooden Breast Syndrome, FOXO1 may be activated to aid diseased birds overcome the issue of hypoxia by preventing symptoms of PH and ascites development.

2.5 Conclusion

The initial intrigue to study the lungs in a disease predominantly affecting muscle was the undeniable, constant trend of hypoxia and low oxygen saturation in muscle tissues and blood seen in broiler chickens affected with Wooden Breast Syndrome. Characteristic of poor oxygen exchange, these trends suggested potential changes in lung morphology, obstruction of air channels, or possibly thickening of surfactant could play a large role in the etiology of Wooden Breast Syndrome.

From my research, it does not appear the lungs are involved in Wooden Breast Syndrome development; rather, many of the changes observed in affected lung tissue compared to lung tissue from unaffected birds can perhaps be attributed to the systemic impact of the disease. In the context of Wooden Breast Disease, 141 DE genes is considered a fairly small gene list, as similar studies of muscle tissue yield approximately 500-1,000 DE genes in broilers affected with Wooden Breast Syndrome. Additionally, studies of muscle tissue from affected broilers show significant increases in immune responses and hypoxia that systemically, can originate

in the muscle and aggravate surrounding tissues, which might explain the presence of inflammatory markers and hypoxia-related DE genes in the lung tissues. A remarkable finding from this research is the clear lipid dysregulation, which has been consistently observed in birds affected with Wooden Breast Syndrome. Although the lungs' role in Wooden Breast Syndrome cannot be completely ruled out, more studies must be conducted to explore the significant pathways and cellular responses identified by the DE gene analysis of affected lung tissues in this research.

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Appendix A

COMPLETE LIST OF DIFFERENTIALLY EXPRESSED GENES

Table 5. **Complete List of Identified DE Genes.** The 141 differentially expressed genes in the table below were identified using Cuffdiff v2.2.1 and are organized alphabetically by gene symbol.

Gene ID	Gene Symbol	Log_2(Fold Change)
ENSGALG00000000302	-	-2.07745
ENSGALG00000000560	-	1.4061
ENSGALG00000002023	-	-1.73603
ENSGALG00000003876	-	-0.891618
ENSGALG00000004734	-	0.760221
ENSGALG00000005180	-	1.4514
ENSGALG00000006172	-	-0.913651
ENSGALG00000007645	-	0.898861
ENSGALG00000010268	-	0.821745
ENSGALG00000010294	-	1.25617
ENSGALG00000010926	-	2.56614
ENSGALG00000013239	-	2.28675
ENSGALG00000014585	-	0.913635
ENSGALG00000015143	-	1.84479
ENSGALG00000016102	-	1.09538
ENSGALG00000016165	-	0.990105
ENSGALG00000016475	-	-0.89431
ENSGALG00000020906	-	1.14594
ENSGALG00000023472	-	0.850476
ENSGALG00000023581	-	2.09108
ENSGALG00000023622	-	1.24825
ENSGALG00000023818	-	4.01504
ENSGALG00000023819	-	2.52068
ENSGALG00000026152	-	0.774396
ENSGALG00000028016	-	1.75672
ENSGALG00000028895	-	2.35355
ENSGALG00000029182	-	0.805494
ENSGALG00000029224	-	-1.85474
ENSGALG00000029411	-	3.63555
ENSGALG00000030033	-	3.24197
ENSGALG00000030700	-	2.4443

ENSGALG00000031037	-	0.962895
ENSGALG00000032304	-	1.15478
ENSGALG00000032781	-	2.93514
ENSGALG00000032897	-	-2.88241
ENSGALG00000033084	-	-1.24092
ENSGALG00000033606	-	-2.72764
ENSGALG00000035921	-	-3.38822
ENSGALG00000036362	-	-1.04888
ENSGALG00000036589	-	3.14304
ENSGALG00000037235	-	-1.57252
ENSGALG00000037916	-	1.17879
ENSGALG00000038375	-	-1.5402
ENSGALG00000038396	-	1.26392
ENSGALG00000038514	-	3.12865
ENSGALG00000039028	-	0.824608
ENSGALG00000039374	-	-
ENSGALG00000040886	-	-0.979053
ENSGALG00000041031	-	1.36789
ENSGALG00000041298	-	0.89905
ENSGALG00000041447	-	-1.1227
ENSGALG00000041555	-	1.15898
ENSGALG00000041600	-	-1.11534
ENSGALG00000042023	-	0.953794
ENSGALG00000043654	-	-0.710123
ENSGALG00000043732	-	4.14212
ENSGALG00000044108	-	3.54392
ENSGALG00000044372	-	-1.68569
ENSGALG00000044418	-	-1.47696
ENSGALG00000044566	-	-0.854981
ENSGALG00000044619	-	1.01208
ENSGALG00000044630	-	-
ENSGALG00000045640	-	0.976962
ENSGALG00000045917	-	0.92956
ENSGALG00000046276	-	-1.79344
ENSGALG00000046533	-	-2.47951
ENSGALG00000032854	ACP5	1.34696
ENSGALG00000040342	ADAMTS1	0.985249
ENSGALG00000001407	ADAMTS15	0.757598
ENSGALG00000005554	ADIPOQ	0.83591
ENSGALG00000035219	ALB	-3.26963
ENSGALG00000007114	APOA1	1.473

ENSGALG00000011784	APOLD1	1.01989
ENSGALG00000009433	BAG3	1.0634
ENSGALG00000008308	BHLHE40	0.926654
ENSGALG00000000141	BLB1	0.855048
ENSGALG00000030038	C3	1.09558
ENSGALG00000028256	CCL19	1.2105
ENSGALG00000008014	CEBPB	0.697107
ENSGALG00000034119	COL15A1	0.948006
ENSGALG00000038311	COL18A1	1.15903
ENSGALG00000027788	CPE	0.89141
ENSGALG00000030866	CPQ	-0.908612
ENSGALG00000014994	CRHBP	-2.35732
ENSGALG00000032143	CRISPLD2	1.04087
ENSGALG00000038393	DMB2	0.807302
ENSGALG00000002335	DOCK3	1.18167
ENSGALG00000005843	EEF1A2	2.38336
ENSGALG00000040971	EGR2	1.3344
ENSGALG00000032746	Enpp2	-0.703766
ENSGALG00000002108	EVPL	0.904402
ENSGALG00000026677	F10	-0.992491
ENSGALG00000030025	FABP4	1.83536
ENSGALG00000015573	FHL5	-0.755287
ENSGALG00000042148	FKBP5	1.09737
ENSGALG00000010316	FRAS1	0.784375
ENSGALG00000034101	FZD5	0.859614
ENSGALG00000015835	GRIK1	-0.977596
ENSGALG00000013548	GZMA	0.898207
ENSGALG00000023436	HDC	-0.885173
ENSGALG00000022586	HPX	1.17621
ENSGALG00000005467	HSD17B2	-2.02338
ENSGALG00000001926	HSPB1	0.682452
ENSGALG00000023772	HSPB7	1.1709
ENSGALG00000007383	HSPB8	0.938039
ENSGALG00000000785	HYAL1	0.698009
ENSGALG00000002138	HYAL2	0.682586
ENSGALG00000004239	IFITM5	1.02797
ENSGALG00000030344	ISYNA1	0.665865
ENSGALG00000035879	ITGA7	0.905429
ENSGALG00000007837	KAZALD1	0.954818
ENSGALG00000026928	KLF4	0.832894
ENSGALG00000031211	Lbx3	1.32283

ENSGALG00000028928	LCAT	0.890919
ENSGALG00000027096	LGALSL	-0.672182
ENSGALG00000030687	LGI3	0.83321
ENSGALG00000015425	LPL	0.853794
ENSGALG00000004530	MMD2	2.19454
ENSGALG00000010983	NPY	1.70619
ENSGALG00000043727	NR4A1	1.0331
ENSGALG00000023881	PLXDC1	0.828196
ENSGALG00000045875	PSCA	0.969037
ENSGALG00000004860	RASD1	0.773946
ENSGALG00000026572	RGS5	0.787132
ENSGALG00000025650	RNaseP_nuc	3.67458
ENSGALG00000034408	SEPP1	-1.50869
ENSGALG00000015568	SH3TC1	-0.684759
ENSGALG00000036125	SHE	0.665525
ENSGALG00000011220	SLC25A29	0.946655
ENSGALG00000004246	SLC6A4	-0.890319
ENSGALG00000017168	SLN	1.20824
ENSGALG00000009415	SMOC1	1.55372
ENSGALG00000003512	SPINK7	1.61248
ENSGALG00000007284	SYNPR	1.6058
ENSGALG00000035075	TAP1	0.79051
ENSGALG00000044442	THBD	0.67959
ENSGALG00000006751	THY1	0.95694
ENSGALG00000017715	TINAGL1	0.698849
ENSGALG00000006106	TNFRSF6B	0.907628
ENSGALG00000015079	UPK1B	0.979939
ENSGALG00000038540	ZBTB16	1.07478

Appendix B

IPA® LIST OF UPSTREAM REGULATORS

Table 6. **List of Activated and Inhibited Upstream Regulators Identified by IPA®.** 103 upstream regulators were shown to either be activated or inhibited based on DE gene analysis. Only genes with activation z scores are shown here.

Upstream Regulator	Molecule Type	Activation z score
TP53	transcription regulator	2.478
Cg	complex	2.434
Insulin	group	2.322
IL4	cytokine	2.22
TREM1	transmembrane receptor	2.213
FOXO1	transcription regulator	2.2
IL1	group	2.186
IFNG	cytokine	2.15
CEBPB	transcription regulator	2.079
IRS1	enzyme	2
HSF1	transcription regulator	1.992
Pkc(s)	group	1.982
IL5	cytokine	1.982
LDL	complex	1.979
EGF	growth factor	1.969
SHH	peptidase	1.964
S100A9	other	1.964
HGF	growth factor	1.964
SREBF1	transcription regulator	1.957
PGR	ligand-dependent nuclear receptor	1.957
VEGFA	growth factor	1.947
BMP2	growth factor	1.94
KLF4	transcription regulator	1.938
Ins1	other	1.935
AR	ligand-dependent nuclear receptor	1.919
IL6	cytokine	1.834
NFkB (complex)	complex	1.761
Growth hormone	group	1.757
PTH	other	1.746

STAT6	transcription regulator	1.715
P38 MAPK	group	1.71
PDGF BB	complex	1.64
IGF1	growth factor	1.581
CREB1	transcription regulator	1.576
Vegf	group	1.526
IL17A	cytokine	1.52
Interferon alpha	group	1.491
PPARG	ligand-dependent nuclear receptor	1.485
NFE2L2	transcription regulator	1.407
AGT	growth factor	1.4
SMARCA4	transcription regulator	1.4
TNF	cytokine	1.366
SP1	transcription regulator	1.355
CSF2	cytokine	1.287
IL1B	cytokine	1.27
BDNF	growth factor	1.268
ERBB2	kinase	1.264
TGFB1	growth factor	1.187
CHUK	kinase	1.172
FOS	transcription regulator	1.172
LIPE	enzyme	1.131
Tgf beta	group	1.109
IL1A	cytokine	1.109
POU5F1	transcription regulator	1.091
CDKN2A	transcription regulator	1.091
Akt	group	1.081
Nfat (family)	group	1.067
GDF2	growth factor	1.067
IL13	cytokine	1.051
CEBPA	transcription regulator	1.05
LEP	growth factor	1.05
FGF2	growth factor	1.045
PPARA	ligand-dependent nuclear receptor	1.037
FSH	complex	1
MIR17HG	other	1
FOXA1	transcription regulator	1
INS	other	0.984
APP	other	0.896
IKBKB	kinase	0.849
HRAS	enzyme	0.842

STAT3	transcription regulator	0.805
RAF1	kinase	0.762
VCAN	other	0.64
NFKBIA	transcription regulator	0.614
PPARGC1A	transcription regulator	0.577
BRCA1	transcription regulator	0.555
ESR1	ligand-dependent nuclear receptor	0.402
OSM	cytokine	0.362
NOS2	enzyme	0.333
SIRT1	transcription regulator	0.294
TP63	transcription regulator	0.272
MYC	transcription regulator	0.219
MAPK1	kinase	0.2
CSF3	cytokine	0.152
ERK1/2	group	0.104
IL10RA	transmembrane receptor	0
JAG2	growth factor	-2
FAS	transmembrane receptor	-1.98
CD3	complex	-1.762
PML	transcription regulator	-1.561
PTEN	phosphatase	-1.352
GATA4	transcription regulator	-1.185
ID3	transcription regulator	-1.123
MYOD1	transcription regulator	-1.029
mir-21	micro RNA	-1
TP73	transcription regulator	-0.952
WNT3A	cytokine	-0.762
HIF1A	transcription regulator	-0.547
FOXA2	transcription regulator	-0.485
ESR2	ligand-dependent nuclear receptor	-0.464
IL10	cytokine	-0.392
HOXA10	transcription regulator	-0.378
PPARD	ligand-dependent nuclear receptor	-0.318