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Potential Roles of mTOR and Protein Degradation Pathways in the Phenotypic Expression of Feed Efficiency in Broilers

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Abstract

The cost of feed represents as much as 70% of the total cost of raising a meat producing animal to market weight. Thus, feed efficiency (FE; g gain: g feed) is a very important genetic trait in animal agriculture. We have observed that a hallmark of low feed efficiency in a highly selected male broiler (meat chicken) line was extensive protein oxidation that probably resulted from increased reactive oxygen species being produced by the mitochondria. Repair or resynthesis of damaged proteins would therefore represent a considerable energetic drain and contribute to the phenotypic expression of low feed efficiency. In the present study, a software program (Ingenuity Pathway Analysis, IPA) facilitated the analysis and interpretation of data from a 4 x 44k chicken oligo array on breast muscle along with data from previous studies obtained from broilers individually phenotyped for FE. The findings support a hypothesis that differential expression of genes associated with the Akt/mTOR, protein ubiquitination, and proteasome pathways through modulation of transcription and protein turnover could play an important role in the phenotypic expression of feed efficiency. Confirmation of this hypothesis will require a thorough assessment of protein expression as well as protein and enzyme activity measurements associated with these pathways in the low and high FE broiler phenotypes.

Keywords: Broilers; Feed efficiency; Microarray; mTORC1; Ubiquitination; Proteosome

Abbreviations: Akt: Protein kinase B; AK: adenylic kinase; CRYAB: crystalline, alpha B; eiF; eukaryotic initiation factors; FE: feed efficiency; FOXO: forked head box O3; Hsp: heat shock proteins; mTOR: mammalian target of rampamycin; PA: phosphatidic acid; PI3K: phosphatidyl inositol 3-kinase; PTEN: phosphatidylinositol 3-phosphatase; PIP3;phosphoinositol 3,4,5 triphosphate; PIP2: phosphoinositol 4,5 diphosphate; Rheb: Ras homolog enriched in brain; Ras: Rat sarcoma proteins; RING: Really interesting new gene; S6K: ribosomal protein s6 kinase beta; TSC1 and 2: tuberous sclerosis 1 and 2; Ub: Ubiquitin; USP5: Ub specific peptidase 5

Introduction

Although massive amounts of microarray data have produced genome-wide analysis of differentially expressed genes in a variety of distinct phenotypes, the translational approaches that can characterize cellular and physiological functionalities of differentially expressed genes have been limited. The use of pathway analysis software, prediction and interpretation of cellular pathways can be an essential process in generating hypotheses and in understanding the functional importance of differentially expressed genes identified by global gene expression analysis.

With severe drought conditions, diversion of grain to ethanol production, and increasing demand for grain globally, feed efficiency (FE, gain to feed) remains one of the most important genetic traits in animal agriculture. Gene expression studies in a male broiler line have revealed that a high FE broiler phenotype exhibited up-regulation of genes in breast muscle associated with signal transduction pathways, anabolism, energy sensing, and energy coordination activities, whereas a low FE broiler phenotype exhibited up-regulation of genes associated with cytoskeletal architecture and/or muscle development, and stress responsive genes including heat shock proteins and superoxide dismutase [1-3].

Protein accretion and degradation processes can influence overall energetic efficiency in an animal due to the size of the skeletal muscle system and expense of energy required for protein synthesis. As such, protein synthesis and protein degradation processes have a major impact on energy usage within a muscle cell. The mammalian target of rapamycin (mTOR) in conjunction with Akt (also known as protein kinase B) are required via the phosphatidyl inositol 3- kinase (PI3K)/Akt/mTOR pathway for skeletal muscle cell development [4-7]. Activation of this pathway leads to rapid phosphorylation of ribosomal protein s6 kinase beta (p70s6K or S6K) and eukaryotic initiation factors (e.g. eiF3, eiF4) that increase mRNA translation and protein synthesis [8,9]. Postnatal growth is associated with upregulation of the PI3K/Akt/mTOR signaling pathway and according to Bodine et al. "... The activation of the PI3K/mTOR/Akt pathway and its downstream targets…. is intimately involved in regulating skeletal muscle fiber size, and ……. can oppose muscle atrophy induced by disuse" [4]. Taken together, these studies indicate that the activation of the Akt/mTOR signaling pathway is critical for regulating skeletal muscle development. Also, it has been reported that the mTOR pathway genes were upregulated in duodenum and liver in broilers with low residual feed intake (high feed efficiency) compared to broilers with high residual feed intake (low feed efficiency) [10].

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Oxidized proteins are targeted by the protein ubiquitination system to repair or degrade proteins to maintain optimal protein functionality. A hallmark of the low FE broiler phenotype is an extensive increase in protein oxidation [11]. In the protein ubiquitination pathway, an enzymatic cascade in the cell first attaches ubiquitin (Ub) to the damaged protein that is then conveyed to proteasomes where proteolysis occurs [12,13]. The 19S proteasome complex has been reported to enhance RNA polymerase II activity and play a role in regulating RNA transcription initiation [13-15]. Since protein synthesis, ubiquitination, and amino acid recycling require considerable energy expenditure, it is reasonable to hypothesize that protein ubiquitination and proteasome expression could play an important role in overall energetic efficiency. Based on the importance of the Akt/mTOR pathway for muscle development [4] and the proteasome-protein ubiquinylation system for protein repair mechanisms [12,13], the major goal of this study was to use pathway analysis to develop hypotheses pertinent to muscle development and protein degradation mechanisms associated with the phenotypic expression of high and low feed efficiency in poultry. These hypotheses were developed from an amalgamation of data from previous studies [1,2,11] in combination with data mining from microarray dataset [1] that has not been previously reported.

Materials and Methods

Male broilers in this study were selected from a group of 100 that were tested for feed efficiency in breeder male replacement stock as previously described [16]. Briefly, birds (6 wk. of age) were individually housed in cages (51 × 51 × 61 cm) at thermoneutral temperature (25°C) with 15 hours of light and 9 hours of dark lighting schedule. Feed intake (from 6 to 7 wk.) and 6- and 7-wk BW were determined to calculate feed efficiency (FE; g gain/g feed intake). From this group of birds, 16 were identified that exhibited the lowest (n = 8) or highest (n = 8) FE within the initial group of 100 males. The birds were color-coded, transported to the University of Arkansas, and housed in similar cages and environmental conditions. Birds were provided access to water ad libitum. All birds received the same corn-soybean based diet (typical of commercial diets in the poultry industry) ad libitum during the feed efficiency trial (20.5% protein, 3,280 kcal/kg). All procedures for animal care complied with the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocol #14012). Birds were killed by an overdose of sodium pentobarbital (i.v.) and breast muscle tissue obtained and flash frozen in liquid nitrogen.

Detailed procedures for isolation of RNA, and microarray hybridization, data collection and analysis has been previously reported by Kong et al. [1]. Briefly, RNA was extracted from breast muscle (pectoralis superficialis) from broilers individually phenotyped for FE that had been flash frozen in liquid nitrogen and stored at -80°C previously [17]. Feed efficiency (body weight gain to feed consumed during the week of feed efficiency phenotyping of individual birds) in the low and high FE phenotypes were 0.46 ± 0.01 and 0.65 ± 0.01, respectively, representing an identical difference (0.19) in FE as that reported by Bottje et al. [16]. RNA samples from high and low FE phenotype tissue (n = 6 per phenotype) were pooled and fluorescently labeled cRNA was generated using a Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA) [1]. The fluorescently labeled cRNA was purified (Qiagen RNeasy Mini Kit, Qiagen Inc., Valencia, CA) and equal amounts of Cy3 and Cy5 labeled cRNA were hybridized on a 4 X 44K Agilent chicken oligo microarray (array ID: 015068) (i.e. four replicates per gene per array). The hybridized slides were scanned using a GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA) with the tolerance of saturation setting of 0.005%.

Global normalization by locally weighted linear regression (LOWESS) was applied to the background-corrected red and green intensities. Genes (array spots) of the 44K array with significant signal intensities were sorted by absolute real (foreground) fluorescent signal >100 and signal to noise ratio (SNR) >3, meaning that real signals of the samples were three times greater than background signals. Differentially expressed genes were identified with a moderated t-statistic and its corresponding p-value based on empirical Bayes methods [18]. The resulting p-values were adjusted for multiple testing by false discovery rate (FDR) [19]. All analysis techniques were implemented in R program (http://www.R-project.org). Genes with an adjusted p-value for FDR below 0.05 were considered statistically different and identified as differentially expressed genes. Results were deposited into the Gene Expression Omnibus (GEO; accession number: GSE24963; http://www.ncbi.nlm.nih.gov/geo). Microarray gene expression was validated previously by comparison with values obtained by qRT-PCR for 33 different genes [1].

Ingenuity Pathway Analysis (http://www.ingenuity.com/) (IPA) software was utilized in this study as a framework to place differentially expressed genes from the microarray dataset into canonical pathways to facilitate interpretation. For example, the Akt/mTOR signaling and ubiquitination canonical pathways along with literature citations provided by the IPA program were used in to identify sets of genes in the microarray data associated with these pathways. Fold differences in gene expression are listed in Tables 1 and 2, and depicted in Figure 1 and 2, respectively. All differentially expressed genes were different ($P< 0.05$) between the high and low FE broiler phenotypes.

Results and Discussion

The Akt/mTOR pathway

Table 1 provides a list of genes and a brief description of their activities that are directly or indirectly related to the Akt/mTOR signaling pathway. The list in Table 1 is grouped by genes that were differentially expressed ($P< 0.05$) between high and low FE phenotypes at greater than 1.3 fold (i.e. expression values differed by 30% or more), and genes that fell between 1.1 and 1.3 fold differences (i.e. 10 to 30% difference in expression between phenotypes). Genes are also shown whose transcribed protein would be activated or deactivated by other genes or compounds and therefore would play an important role in the Akt/mTOR signaling pathway. In this discussion, it needs to be made clear that activation or inactivation of protein intermediates in the pathway is being extrapolated from mRNA expression; definitive confirmation of these hypothesized mechanisms will require additional protein expression analysis and/or measurement of protein activity.

Figure 1, that depicts mRNA expression data in Table 1, indicates; a) that mTORC1 was up-regulated in the high FE broiler phenotype, and b) that mTORC1 would be more active due to the inhibitory effect of Akt of tuberous sclerosis 1 and 2, (TSC1, TSC2). The activity of mTORC1 would also be enhanced by phosphatidic acid (PA) and AMP-mediated activation of Rheb (Ras homolog enriched in brain). As discussed below, increased mTORC1 activity would enhance protein and ribosome biosynthesis as well as muscle hypertrophy through increased activities due to eukaryotic translation initiation complex, S6K, and the 40S ribosome. Precedence for involvement of the mTOR pathway in the phenotypic expression of high FE has been observed in liver and duodenal tissue in broilers divergently selected for residual feed intake [10].

Table 1: Genes in breast muscle of the high feed efficiency (FE) broiler phenotype associated with the mTORC1 complex signaling pathway. All genes were in sections A, B, and C were differentially expressed (P < 0.05) at the fold differences that are presented. Genes in section D were not differentially expressed. A graphical depiction of the pathway is shown in Figure 1.

Abbreviation	Gene Name (Fold Diff)	Brief Description – Cellular role
A) Genes up-regulated (P < 0.01) in the high FE phenotype (> 1.30)		
AMPK*	AMP-activated protein kinase (1.31)	- increases phosphorylation of TSC2 [35]
Chemokine	Chemokine (1.48)	- a family of cytokines that activateAkt[20]
mTORC1	mammalian target of rapamycin complex 1 (1.52)	- phosphorylates S6K1, promotes Akt/mTOR signaling & cell growth [52]
		- mTORC1 inhibition decreases muscle mass and metabolism [7]
PI3K *	1-phosphatidylinositol 3-kinase (1.31)	- catalyzes PIP2 conversion to PIP3 with activation of Akt, [21]
PLC	phospholipase C (1.60)	- catalyzes conversion of PIP2 to DAG (+ IP3) [22]
pr1 (pPIK3RI)*	PPI3K regulatory subunit (1.42)	- activation of Akt[21]
p85 (pik3r)*	PI3K p85 subunit (1.31)	- increases activation of PI3K [23]
Ras*	Rat sarcoma proteins (1.30)	- increases activity of S6K [53]
		- phosphorylation of Akt[24]
		- phosphorylation of PI3K [54]
B) Genes up-regulated (P < 0.05) in the high FE phenotype (between 1.10 to 1.29 fold)		
AK	adenylic kinase (1.20)	- catalyzes the conversion of ADP to AMP [55]
eIF3	eukaryotic elongation initiation factor 3 (1.17)	- reacts with 40S Ribosome [56]
PDK1	phosphoinositide kinase 1 (1.21)	- increases phosphorylation of Akt[57]
PLD	phospholipase D (1.12)	- increases hydrolysis of PC and catalyzes conversion of PC to PA [58]
PTEN	phosphatidylinositol-3-phosphatase (1.23)	- increases dephosphorylation of PIP3 [37]
RSK	ribosomal s6 kinase (1.20)	- increases inactivation of TSC2 protein [59]
S6K	ribosomal protein S6 kinase beta (1.15)	- increases activation of RPS6 protein [60]
40S ribosome	40S ribosome (1.20)	- formation of complex with eIF3 [56]
C) Genes down-regulated (P < 0.05) in the high FE phenotype (> 1.30 fold)		
Myostatin	Myostatin (1.35)	- down regulation of Akt/mTOR signaling [61]
		- increased binding of Smad 3 [62]
D) Non differentially expressed genes.		
Akt	also known as Protein Kinase B	- phosphorylation of TSC complex; decreases TSC-mediated inhibition of mTORC1[35]
FOXO	Forkhead box O3	- protein and cellular component degradation by FOXO is inhibited by Akt-mediated repression of FOXO transcription factors [13,32]
mTORC2	mammalian target of rapamycin protein complex 2	- increases phosphorylation of Akt (protein kinase B) [28]
Rheb	Ras homolog enriched in brain	- increases activation of mTORC1 [34]
Smad 2/3	small body size gene and mothers against decapentaplegic	- inhibition of Akt[31]
TSC1-TSC2	tuberous sclerosis complex –	- inhibition of active Rheb protein; tumor suppressor proteins [25]

*Upregulation of these genes in breast muscle of the high FE phenotype was previously reported [2].

Bodine et al. [4] demonstrated that the Akt/mTOR pathway is required for muscle hypertrophy, and that genetic activation of Akt causes muscular hypertrophy in vivo and opposes muscle atrophy. Although Akt was not differentially expressed, Akt activity would likely be enhanced in the high FE phenotype muscle due to increased mRNA expression of a) PDK1, b) chemokine complex [20], c) 1-phophatidyl¬inositol 3-kinase (PI3K) [21] and PI3K regulatory subunits PPIK3RI and p85 (pik3r) [22,23], and d) Rat sarcoma (Ras) proteins[24].

Akt indirectly stimulates mTORC1 activity by inhibiting TSC 1- and TSC2-attenuation of Rheb activation of mTORC1 [25,26]. Downregulation of myostatin in the high FE phenotype would also favor muscle hypertrophy by activating Akt. Demonstration of a role of myostatin as a negative regulator of muscle growth was provided by McPherron et al. [27]. Muscle hypertrophy following myostatin inhibition has been demonstrated in three ways; in a knockout model of myostatin, with a myostatin antagonist (follistatin), and with anti-myostatin antibodies [28,29]. The ability of myostatin to attenuate muscle growth is mediated by binding to the activin receptor IIB that in turn leads to Smad 2- and Smad3-inhibition of Akt [30,31].

Furthermore, Smad-mediated inhibition of Akt activity is blocked by mTOR/mTORC1 [30,31]. Thus, mTORC1 would be further stimulated by a feed forward type mechanism in which an increase in Akt activity would diminish the negative effects of the TSC complex on Rheb activity [25,26]. Akt also acts to inhibit FOXO (forkhead box O) that increases protein degradation mechanisms in the cell [32]. With regard to energetic efficiency, FOXO is also involved in degradation of cellular components, particularly mitochondria [13,33]. Thus, increased Akt activity by mechanisms outlined above could have an important effect in maintaining mitochondrial numbers and/or function in the high FE broiler phenotype.

Several genes that were upregulated between 10 and 30% (1.1 to 1.3 fold) in the high FE phenotype (Table 1B) could also stimulate mTORC1 complex activity. For example, adenylic kinase (AK) converts ADP to AMP that in turn increases Rheb-mediated activation of mTORC1 [34]. By diminishing TSC complex inhibition of Rheb activity, ribosomal s6 kinase (RSK) would also increase mTORC1 activity [35]. Phosphatidic acid (PA) has also been reported to increase mTORC1 activity [36]. As such, the combined activities of phosphatidylinositol 3-phosphatase (PTEN) that converts phosphoinositol 3,4,5 triphosphate (PIP3) to phosphoinositol 4,5 diphosphate (PIP2) [37] and phospholipase C (PLC) [22] would increase diacyl glycerol (DAG) as shown in Figure

Table 2: Genes in breast muscle of the high feed efficiency (FE) broiler phenotype associated with the protein ubiquitination and RNA polymerase II pathways. All genes were differentially expressed (P < 0.05) at the fold differences that are presented. A graphical depiction of the pathway is shown in Figure 2.

Abbreviation	Gene Name (Fold Diff)	Brief Description – Cellular role
A) Genes up-regulated (P < 0.01) in the high FE phenotype (> 1.30)		
19S	19S proteasome (1.34)	- protein ubiquitination pathway [13,43]
		- stimulation of RNA II polymerase complex [14,48]
E2A	Ub1 conjugating enzyme E2A (1.40)	- protein ubiquitination, conveys ubiquitinated protein to EC3 [13]
RNA Pol II	γsubunit of transcription initiation factor IIA (1.30)	-component of RNA polymerase II initiation complex [14]
TFIIH	general transcription factor IIH (1.38)	- component of RNA polymerase II initiation complex [14]
B) Genes up-regulated (P < 0.05) in the high FE phenotype (between 1.10 to 1.29 fold)		
EC3	Ub protein ligase E3 component n-recognin1 (1.25)	- conveys ubiquitin conjugate to 19S/26S proteasome [13]
TFIIA	general transcription factor IIA (1.25)	- component of RNA polymerase II initiation complex [14]
C) Genes down-regulated (P < 0.05) in the high FE phenotype (> 1.30 fold)		
CRYAB	crystalline, alpha B (member Hsp family) (1.34)	- member of heat shock protein family; chaperone and refolds proteins [43,47]
E2E2	Ub conjugating enzyme E2E2 (1.37)	- protein ubiquitination, conveys ubiquitinated protein to EC3 [13]
HSP90*	heat shock protein 90 kDaα (1.61)	- molecular chaperone, protein repair, move damaged proteins to proteasome [43]
USP5*	Ub specific peptidase 5 (1.33)	- deubiquitination of proteins and peptides [12]

1 Ubiquitin abbreviation = Ub
*Upregulation of these genes in breast muscle of the high FE phenotype was previously reported [1].

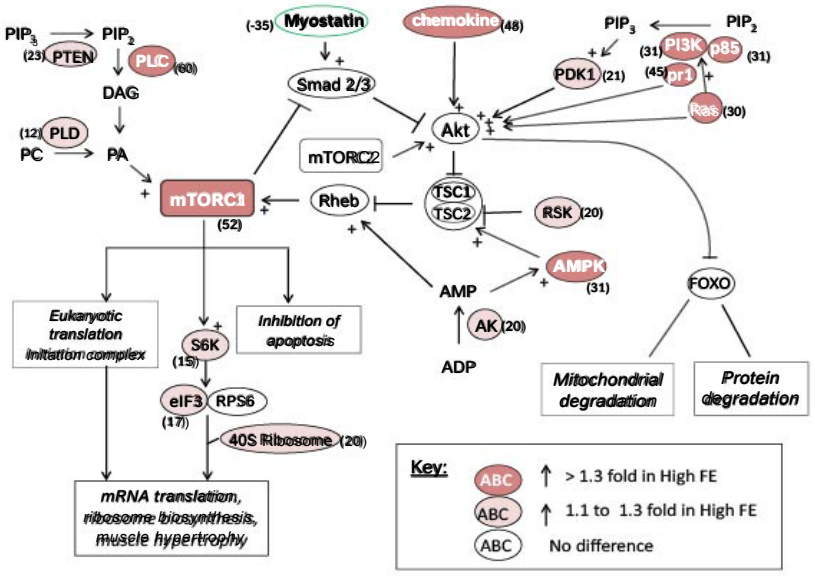

Figure 1: Genes associated with the Akt/Mtor signaling pathway in breast muscle of the high and low feed efficiency broiler phenotypes. Genes in the high FE phenotype are shown in a) red figures with white lettering (dark grey with white lettering) were upregulated by 1.3 fold or more (greater than 30%), b) pink figures with black lettering (light grey with black letters) were upregulated between 1.1 and 1.3 fold (between 10 to 30%) compared to the low FE phenotype (all were significantly different, P < 0.05). Myostatin was down-regulated 1.35 fold (35%) in the high FE phenotype compared to the low FE phenotype. Numbers in parentheses indicate the percentage of up- or down-regulation of each gene with respect to the low FE phenotype. Genes or compounds that are in black type in a figure with a white background were not differentially expressed but represent important intermediary steps in the Akt/mTOR pathway.

Abbreviations(in alphabetical order): ADP (adenosine diphosphate), AK (5'-AMP kinase, adenylic kinase), Akt (protein kinase B), AMP (adenosine monophosphate), AMPK (AMP kinase), DAG (diacylglyceride), eiF3(eukaryotic translation initiation factor 3), FOXO (forkhead box O), mTORC1 (mammalian target of rapamycin [mTOR]complex 1), mTORC2 (mTOR complex 2), PA (phosphatidic acid), PC (phosphatidyl choline), PDK1 (3-phosphoinositide dependent protein kinase-1), PIP2 (1-phosphatidyl-D-myo-inositol 4,5-bisphosphate), PIP3 (phosphatidylinositol-3,4,5-trisphosphate), PI3K (1-phosphatidylinositol 3-kinase), PLC (phospholipase C), PLD (phospholipase D), PRAKG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit),PTEN(phosphatidylinositol-3-phosphatase),TSC1-TSC2 (tuberous sclerosous 1&2), Ras (rat sarcoma), Rheb (Ras homolog enriched in brain), 40S ribosome (ribosomal 40S subunit), RSK (ribosomal s6 kinase), S6K (Ribosomal protein S6 kinase beta), Tpm3 (tropomyosin like 3).

1. Finally, a transcriptional factor (TFIIIC$_\varepsilon$) was also upregulated (1.3 fold) in the high FE phenotype (not shown). TFIIIC$_\varepsilon$ is one component in the assembly of the RNA polymerase III initiation complex required for synthesis of 5S rRNA [38] and the binding of mTOR to TFIIIC$_\varepsilon$ that removes an inhibitory factor of the mTOR signaling pathway [39]. Thus, up-regulation of TFIIIC$_\varepsilon$ could also stimulate mTOR activity in the high FE phenotype.

The RSK-mediated inhibition of TSC, however, is attenuated via AMP-activated protein kinase (AMPK) [35]. As indicated in Table

1, AMPK mRNA expression was elevated in the high FE broiler phenotype [2]. Elevated expression of AMPK would increase ATP levels in the cell by inhibiting synthetic pathways (e.g. gluconeogenesis, fatty acid and protein synthesis) and enhancing catabolic pathways [40]. Inhibition of protein synthesis would appear to be detrimental to the high FE phenotype. However, AMPK has also been associated with mitochondrial biogenesis, possibly through activation of PGC1-$_{\alpha}$ and silencing of AMPK expression in endothelial cells was also associated with diminished antioxidant protection and with increased apoptosis [41]. Therefore, these beneficial components of AMPK activity must outweigh the possible negative effects of lowered protein synthesis in the high FE phenotype.

Protein ubiquitination and RNA polymerase II activity

Table 2 lists genes associated with the protein ubiquitination pathway and the impact that the 19S proteasome protein complex has on RNA polymerase II activity. The genes in Table 2 are shown graphically in Figure 2. Briefly, the protein ubiquitination pathway 'tags 'damaged proteins with ubiquitin (Ub) that are directed to proteasomes for proteolysis and recycling of amino acids for protein resynthesis [12,13,42,43]. In this pathway, damaged or misfolded proteins are conjugated with Ub, a 76 amino acid long protein, that is then conveyed to a 26S proteasome complex. Ub is typically found in a linear Ub chain or bound to specific ribosomal protein subunits rather than as individual Ub molecules [13].

Ub is added to proteins to form a protein-conjugate with 5 Ub molecules via an enzymatic cascade consisting of the following; a Ub like modifier activating enzyme (E1) requiring input of ATP, a group of Ub-conjugating enzymes (E2) that serve as Ub-carrier proteins, and a group of Ub ligating enzymes (EC3) Figure 1 and 2 of [13]. Ub specific peptidase (USP5) performs a role in releasing ubiquitin from ubiquitin-conjugated peptides following proteolysis that can then be used for further protein ubiquination.

There was no difference in mRNA expression of E1 but there was both an up-regulated E2 (E2A) and a down-regulated E2 (E2E2) in breast muscle of the high FE phenotype compared to the low FE phenotype (Table 2, Figure 2). Specificity in protein degradation is achieved in the second step of protein ubiquitination by the large number of different E2s present in the cell [13]. The significance of both up-regulated and down-regulated E2 enzymes in the high FE phenotype in this study is not apparent, but may play a role in turnover of specific types of damaged or misfolded proteins. In the third step of protein ubiquitination, the EC3 enzyme transfers Ub moieties to a lysine on the target protein. There are two general groups of E3 ligase enzymes that are either designated HECT (homologous to E6-AP carboxy-terminus) E3 or RING (really interesting new gene) E3 [42]. The RING proteins contain groups of cysteine and histidine moieties that bind zinc and most of the E3 enzymes fall under the category of RING E3s [42]. In general, E3 enzymes provide a scaffold structure to bring the protein and E2 enzyme close together that is a requisite for optimal ubiquitin

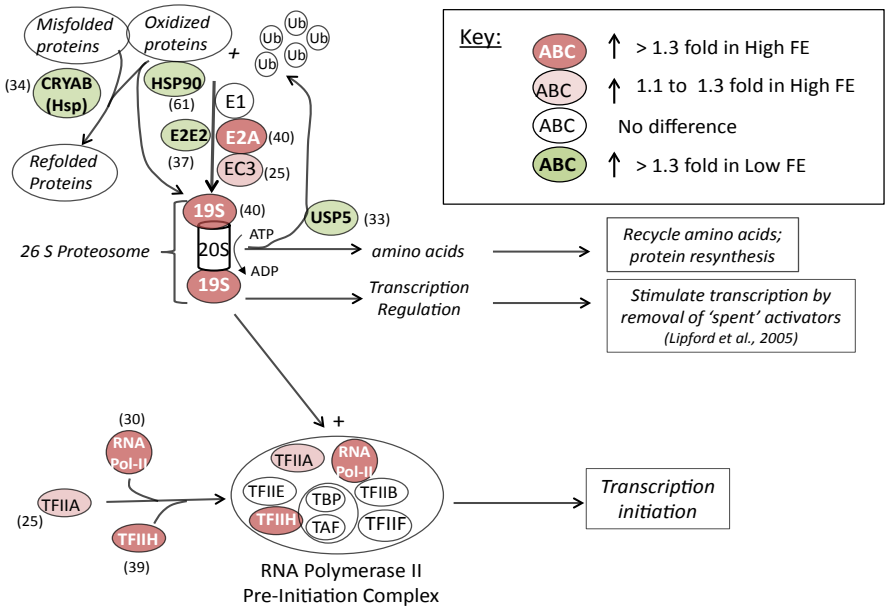

Figure 2: Genes associated with the protein ubiquitination pathway in breast muscle of the high and low feed efficiency broiler phenotypes. Genes in the high FE phenotype are shown with; a) solid red figures with white lettering (dark grey with white lettering) were up-regulated by 1.3 fold or more (greater than 30%), and b) pink figures with black lettering (light grey background with black letters) were upregulated between 1.1 and 1.3 fold (10 to 30%) (> 1.3 fold; more than 30%) or pink (between 1.1 and 1.3 fold, or between 10 to 30%) compared to the low FE phenotype (all were significantly different, $P < 0.05$). Genes in the low FE phenotype are shown with a green background with black lettering (grey with black lettering) that were upregulated by 1.3 fold or more (greater than 30%) in the high FE phenotype compared to the low FE phenotype. Numbers in parentheses indicate the percentage of up-or down regulation of each gene with respect to the low FE phenotype. Genes or compounds that are in black type with an open circle were not differentially expressed but are important intermediary steps in a given pathway.

Abbreviations(in alphabetical order);19S (19S proteasome), 20S (20S proteasome), 26S proteasome (composed of two 19 S and 1 20S proteasome complexes), CRYAB (crystallin, alpha B, member of heat shock protein [Hsp] family), E1 (ubiquitin like modifier activating enzyme 1), E2A (ubiquitin-conjugating enzyme E2A), E2E2 (ubiquitin conjugating enzyme E2E 2), EC3 (ubiquitin protein ligase E3 component n-recognin 1), HSP90 (heat shock protein 90k Da□□cytosolic) class B), RNA Pol-II (gamma subunit of transcription initiation factor IIA), RNA polymerase II), TAF (TATA box binding protein-associated factor) , TBP (TATA box binding factor), TFIIA (TFIIH (general transcription factor IIH), TFIIB (general transcription factor IIB),TFIIE (RNA polymerase-II pre-initiation complex factor),TFIIF (general transcription factor IIF), Ub (Ubiquitin polypeptide), USP5 (ubiquitin specific peptidase 5, deubiquitinylating enzyme),

conjugation [44,45]. The EC3 RING enzyme group was up-regulated ($P < 0.05$) by 1.25 fold in the high FE compared to the low FE phenotype (Table 2).

In the next step of proteolysis, E3 ligases convey the Ub-protein to the 26S proteasome that contains three multi-protein complexes; a single 20S proteasome with 19S proteasomes on either end [43,46]. The 26S proteasome functions to isolate the process of proteolysis to a very small (nanometer size) structure within the cell thereby preventing indiscriminate protein degradation and insures that only certain proteins will be degraded [13]. For proteolysis, the ubiquitinylated protein is transferred to the 19S proteasome that has a 'lid' component and provides a linearized protein to the inner core of the 20S proteasome where proteolysis occurs with expenditure of ATP [14,43]. As indicated previously, Ub released from proteins or peptides following the activity of the Ub specific peptidase 5 (USP5) are then reused for protein ubiquitination. In the current study, USP5 was down-regulated in the high FE phenotype and therefore up-regulated in the low FE phenotype (Table 2). In previous studies, we reported a pervasive increase in protein carbonyl levels (protein oxidation) in several tissues along with higher levels of Ub in breast muscle obtained from the low FE phenotype [11]. Thus, it is not surprising that USP5 would be up-regulated in the low FE broiler phenotype in the present study as this could aid in continual repair of oxidatively damaged proteins. Since both repair and re-synthesis of proteins requires considerable energy expenditure, this mechanism could play a major role in cellular inefficiency in the low FE broiler phenotype.

Damaged proteins can also be conveyed directly to the 19S proteasome by conjugation with molecular chaperones [47] that could include heat shock proteins [43]. As shown in Table 2 and Figure 2, HSP90 as well as crystalline, alpha B (CRYAB) which is a member of the heat shock protein (Hsp) family, were also up-regulated in the low FE phenotype. As indicated previously [1], increased expression of heat shock proteins is further indication of increased oxidative stress in the low FE broiler phenotype. Repair of proteins only slightly damaged or misfolded, may be accomplished by interaction with heat shock proteins [43].

19S Proteosome, transcription, and RNA-polymerase II activity

The 19S proteasome has also been shown to exert effects on transcription by quickly removing activators of gene transcription and directly stimulating RNA polymerase II/RNA polymerase II pre-initiation complex activity [14,15,48]. By removing activators from promoter regions, transcription can be enhanced, and possibly made more efficient. The 19S proteasome may also increase RNA polymerase II activation directly [14]. As shown in Table 2 and Figure 2, several genes in the RNA polymerase II pre-initiation complex, TFIIA, RNA-polymerase II, and TFIIH were all up-regulated in the high FE phenotype. Thus, greater transcriptional activity may be enhanced in the high FE phenotype.

Summary

In this study, we have used pathway analysis to develop hypotheses regarding muscle development and protein degradation mechanisms in the phenotypic expression of feed efficiency in poultry. With increased global demand for high quality animal protein, it is important to make efficient uses of resources including the use of grains in animal production. We recognize that the expression of several genes described in this study could be dismissed by many readers as exhibiting a marginal differential expression between the low and high FE phenotypes; especially for those genes that were only 10 to 30% different between groups. However, the goal of this paper is meant to present a hypothesis and is not meant to be a definitive answer and hope that this might be a utilized by other researchers as well. It should be recognized that the feed efficiency model utilized here and in previous studies [1,2,16,17] investigated healthy animals from the same genetic line and maintained in an ideal environment; i.e. the animals were not challenged by disease or from different genetic lines where large differences in gene expression are expected. Therefore, it is not surprising that subtle differences in gene expression may very well have a major impact in producing the high and low FE broiler phenotypes. For this reason, it may be necessary to use even more sophisticated methods of investigating these phenotypes such as the differential wiring analysis and identification of regulatory and phenotypic impact factors described by Hudson and co-workers [49-51]. In this analytical approach, the connectivity of genes are determined that identifies those genes that may have large contributions in the design of a given animal phenotype even though the gene may not be considered or 'seen' by researchers as being important for a phenotype due to a lack of significance in magnitude of differential expression.

Confirmation of increased activity of the Akt/mTOR pathway and the role of the protein ubiquitination pathway in the high FE phenotype will require additional investigation; e.g. protein expression and protein activity measurements. Mechanistic studies could also be accomplished with gene knockdown models, but these are currently not available in poultry. Nonetheless, this study does provide a strong basis for hypothesizing that Akt/mTOR signaling and protein ubiquitination pathways could play important roles in the phenotypic expression of feed efficiency. The fact that similar results for genes of the mTOR pathway have been observed in a different feed efficiency broiler model (i.e. broilers divergently selected for RFI) and in different tissues (liver and duodenum) [10], strengthens our hypothesis that the Akt/mTOR pathway is important in the phenotypic expression of feed efficiency in broilers. Increased mTORC1 activity may also enhance muscle hypertrophy and compensate for decreased expression of a large number of muscle fiber/cytoskeletal architecture genes that have been previously reported in the high FE phenotype [1,2].

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