Transcriptomic analysis of the liver in aged laying hens with different eggshell strength

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ABSTRACT Eggshell is composed of a very ordered and mineralized structure and is important for egg quality. Eggshell strength is particularly important because of its direct association with economic outcomes and egg safety. Various factors related to laying hens and their environment affects eggshell strength. However, the molecular mechanisms of liver functions related to decreased eggshell strength of aged laying hens are largely unknown. Therefore, this study aimed to identify potential factors affecting eggshell strength in aged laying hens at the hepatic transcriptomic level. A total of five hundred 92-wk-old Hy-line Brown laying hens were screened to select those exhibiting the greatest variation in eggshell strength. Based on the final eggshell strength, 12 hens producing eggs with strong eggshell strength (**SES**) and weak eggshell strength (**WES**) were finally selected (n = 6) for liver tissue sampling. The RNA-sequencing was performed to identify differentially expressed genes (**DEGs**) between the 2 groups. We identified a total of 2,084 DEGs, of which 1,358 genes were upregulated and 726 genes were downregulated in the WES group compared with SES group. According to the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, the DEGs indicated the mammalian target of rapamycin signaling pathway, the Janus kinase-signal transducer and activator of transcription pathway, the mitogen-activated protein kinase signaling pathway, and the insulin resistance pathways. Genes related to fatty liver disease were upregulated in WES group compared with SES group. In addition, expression of several genes associated with oxidative stress and bone resorption activity was altered in aged laying hens with different eggshell strength. Overall, these findings contribute to the identification of genes involved in different intensity of eggshell strength, enabling more understanding of the hepatic molecular mechanism underlying in decreased eggshell strength of aged laying hens.

Key words: aged laying hen, eggshell strength, liver, transcriptome

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INTRODUCTION

Egg quality is determined based on internal and external characteristics (Jacob et al., 2000). Eggshell strength is considered the most important quality among external qualities because of its direct association with economic outcomes and egg safety (Gole et al., 2014). Thus, many poultry scientists have a large attention on an improvement in eggshell strength of laying hens by environmental and nutritional managements.

Eggshells have a highly ordered and mineralized structure composed of an organic component that includes the matrix, eggshell membrane, mammillary knob, and cuticle layer as well as an inorganic component, comprised largely of calcium (**Ca**) carbonate, which accounts for approximately 95% of the weight of the eggshell (Qiu et al., 2020). Several animals (e.g., age, genetics, and diseases) and environmental factors (e.g., stress, diets, and raising facility) are known to affect eggshell formation and, subsequently, eggshell strength (Roberts, 2004; Solomon, 2010). Among these factors, age is the one of the most influential factors. It is well-recognized that eggshell formation cannot fully support the increase in egg size associated with the aging of laying hens (Washburn, 1982; Park and Sohn, 2018). Moreover, it is also likely that the ability of eggshell formation is decreased by increasing ages of laying hens (Gu et al., 2021). This decreased eggshell strength with age of laying hens is often deteriorated by other additional factors impairing eggshell strength such as environmental stress and disease outbreaks (Solomon, 2010). A lot of efforts have been made to ameliorate decreased eggshell strength of aged laying hens, especially by dietary manipulations. Increasing supply or modified supply ratios of Ca, phosphorus (**P**), and other potential minerals such as zinc and magnesium (**Mg**) in diets have been widely

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practiced to improve eggshell strength of aged laying hens (Frost and Roland, 1991; Cufadar et al., 2011; An et al., 2016; Yu et al., 2020; Belkameh et al., 2021). However, the results have been variable, which may be attributed to the limited knowledge of altered nutrient metabolism with age of laying hens.

The application of transcriptomic analysis in poultry science is recently increasing because this analysis can provide a useful information for the molecular changes based on the identification of vital genes and their expression levels, which are related metabolic pathways for poultry raised under different environment and managements (Coble et al., 2014; Li et al., 2015a; Li et al., 2017; Wan et al., 2017). Several previous studies have performed transcriptomic analysis to reveal the molecular mechanisms related to eggshell strength of laying hens with an emphasis of uterine tissues where eggs are actually formed (Sah et al., 2018; Feng et al., 2020). According to the uterine transcriptomic results from laying hens with different intensity of eggshells, Zhang et al. (2019) reported that differences in gonadal hormones in the initial calcification stage of eggshells were related to eggshell strength of laying hens (Zhang et al., 2019). However, there is still a lack of molecular information for the other pivotal tissues in the body of laying hens.

The liver plays a role in various key metabolic functions (carbohydrate, protein, lipid metabolism, bile secretion, etc.), immune response, and detoxification processes (Zaefarian et al., 2019). Thus, maintaining liver health and functions is imperative to support the proper egg production rate and high egg quality of laying hens (Lera, 2018). However, the liver health is generally decreased with age of laying hens. For instance, increasing de novo fat synthesis in the liver of aged laying hens frequently induces an increasing occurrence of fatty liver diseases, which is highly associated with decreased egg production and egg quality (Galea, 2011). However, other molecular mechanisms for liver functions related to decreased eggshell strength of aged laying hens are still largely unknown. In addition, to our knowledge, no hepatic transcriptomic analysis has been performed to investigate novel genes and metabolic pathways of aged laying hens with the different intensity of eggshell strength.

Accordingly, the present study aimed to compare hepatic transcriptomes between aged laying hens with different intensity of eggshell strength based on RNA-sequencing (**RNA-seq**) analysis and to identify the differentially expressed genes (**DEGs**) regarding hepatic molecular functions related to eggshell strength of aged laying hens.

MATERIALS AND METHODS

Animals and Tissue Collection

All experimental procedures were reviewed and approved by the Animal Care and the Use Committee at Chung-Ang University (IACUC: 2018-00002). A total of five hundred 92-wk-old Hy-Line Brown laying hens were used to investigate the eggshell strength at the start of the current experiment. All hens were placed in individual cages (37 × 30 × 40 cm) and raised according to the recommendation of environment and feeds in Hy-Line Brown laying hens (Hy-Line, 2016). The eggshell strength of all eggs produced from 500 hens was measured using the Texture analyzer (model TAHDi 500, Stable Micro System, Godalming, UK) for 10 d. After assessment of initial eggshell strength, 200 hens (100 laying hens with strong eggshell strength and 100 laying hens with weak eggshell strength) were separately selected. The eggshell strength of all eggs produced from the 200 selected laying hens was continuously re-examined for 7 d. A total of 40 hens (20 laying hens with strong eggshell strength and 20 laying hens with weak eggshell strength) were selected again. Within each group of eggshell strength, 6 hens were finally selected based on an additional 6 d of eggshell strength measurements and were assigned to strong eggshell strength (**SES**) and weak eggshell strength (**WES**) group. In addition to eggshell strength, eggshell thickness, and eggshell color of eggs from the SES and WES groups were also measured using a dial pipe gauge (model 7360, Mitutoyo Co., Ltd., Kawasaki, Japan) and the eggshell color fan (Samyangsa, Kangwon, Korea), respectively. All 12 hens were euthanized by CO_2 asphyxiation at 6 h after their oviposition, which is considered the initiation period of calcification in the uterus (Zhang et al., 2019). The liver was rapidly harvested and immediately frozen in liquid nitrogen for RNA-seq analysis. All liver tissue samples were stored at −80°C until further RNA extraction analysis. The detailed procedure of hen selection and liver sampling is presented in Figure 1.

RNA Extraction, Library Construction, and Sequencing

Total RNA was extracted from the liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) after grinding the frozen liver sample under liquid nitrogen condition. A total of 12 RNA integrities were checked using an Agilent Technologies 2100 Bioanalyzer and RiboGreen dye (Invitrogen) and quantified using a Trinean DropSense96 spectrophotometer (Trinean, Gentbrugge, Belgium). Quality control criteria were set as the ratio of 28S:18S > 1 and RNA integrity number >7. Only high quality RNA extracts were used for library preparation. As a result, 8 of 12 RNAs were passed and subjected to RNA-seq (SES = 4 samples and WES = 4 samples). The RNA-seq libraries were synthesized from total RNA using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA). PolyA+ RNA was purified from total RNA using AMPure XP beads and fragmented to 200 to 700 bp (average 350 bp). A total of 8 cDNA was synthesized using reverse transcriptase (SuperScript II, Invitrogen) and random primers. Strand-specific RNA-seq libraries were prepared by following a modified deoxy-UTP strand-

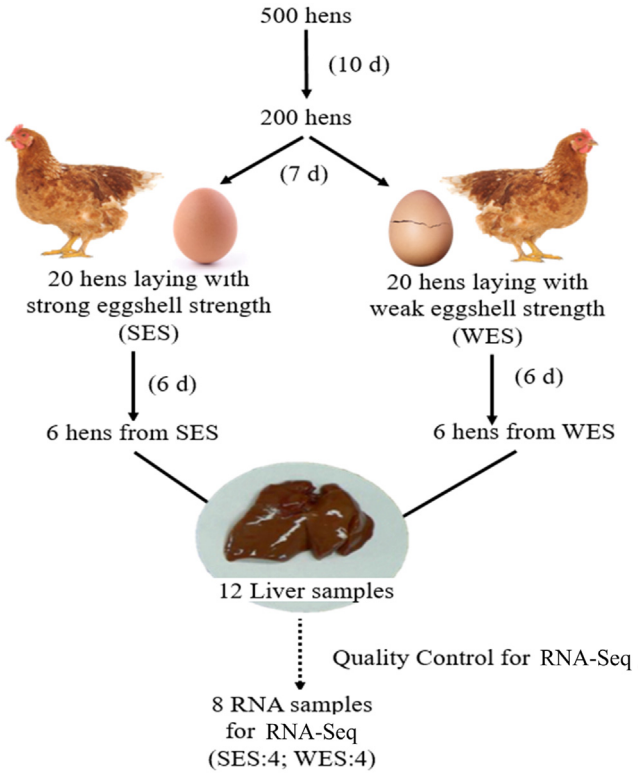

Figure 1. The diagram of the experimental strategy for RNA-sequencing (RNA-Seq). The day in the blank means the recording period of eggshell strength. Six hens from each group were selected for RNA-Seq. However, during the quality control process, 2 samples from strong eggshell strength (SES) group and 2 samples from weak eggshell strength (WES) group were removed, and thus, were not subject to RNA-seq analysis.

marking protocol. The mRNA was used to synthesize double-stranded cDNA. This double-stranded cDNA was subsequently ligated to the barcoded Truseq adapters. Library construction was performed by PCR on the size selected fragments. The resultant library was sequenced on an Illumina Nextseq 500 platform (Illumina) using a paired-end run (2 × 75 bases).

Transcriptomic Analysis

Quality of the raw reads was assessed using FastQC (Andrews, 2010). The raw reads containing adapters or low-quality reads were trimmed using Trimmomatic ver 0.38 (Bolger et al., 2014). The remaining high-quality reads were aligned to the chicken reference genome (GRCg6a, GCA_000002315.5) of the Ensembl genome browser (http://www.ensembl.org/Gallus_gallus/) using HISAT2 ver 2.1.0 (Kim et al., 2015). The mapped reads were quantified based on the exons in the Gallus gallus genome GTF (Ensembl ver. 100) using the featureCounts ver. 1.6.3 of the subread package (Liao et al., 2014). Total DEGs analyses for the acquired raw counts were counted using R package edgeR ver. 3.26.5 and normalization of the raw counts was carried out using the trimmed mean of M-value method (Robinson et al., 2010; Robinson and Oshlack, 2010). Genes with a false discovery rate (**FDR**) < 0.05 and an absolute $\log_2$ fold-change (**FC**) ≥1 were identified as DEGs. The DEGs obtained from WES group were compared with those obtained from SES group. The sequencing data were submitted to the NCBI Sequence Read Archive database under the accession number PRJNA728451.

Multidimensional scaling (**MDS**) was conducted to prove the similarities among samples. Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis were conducted using the Database for Annotation, Visualization and Integrated Discovery (**DAVID** ver 6.8; Dennis et al., 2003). The GO enrichment analysis was performed with biological process (**BP**), cellular component (**CC**), and molecular function (**MF**).

Protein–Protein Interaction Network Analysis and Modules Selection

The Search Tool for the Retrieval of Interacting Genes (**STRING**) database was performed to acquire protein-protein interaction (**PPI**) data. The cutoff DEGs based on an absolute value of $\log_2$FC of ≥1.5 were mapped to STRING measuring the interactive relationship. The PPI network of the cutoff DEGs was visualized by Cytoscape (ver 3.8.2; http://www.cytoscape.org/). A high confidence score (more than 0.9) was considered significant and then disconnected nodes were finally removed. The CytoHubba app in Cytoscape was performed to analyze the hub genes using 5 centrality calculation methods, including Degree, EPC, EcCentricity, MCC, and MNC (Liu et al., 2018). The Molecular Complex Detection (**MCODE**) app in Cytoscape was utilized to investigate the modules of the PPI network. The criteria settings of MCODE were as follows: degree cutoff = 2, node score cutoff = 0.2, k-core = 2, maximum depth = 100 (Yang et al., 2020). Furthermore, the interesting genes derived from these 4 calculation methods, and the function and pathway enrichment analyses were conducted for interesting genes in the modules.

Validation of Differentially Expressed Genes Identification by Reverse Transcription Polymerase Chain Reaction

Nine mRNAs identified as differentially expressed between SES and WES groups in the RNA-seq were selected for verification based on the relative quantification by reverse transcription polymerase chain reaction (**RT-PCR**). The total RNA was used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Waltham, MA) according to the manufacturer's guidelines.

All primer sequences for RT-PCR were designed using Primer-BLAST in the NCBI and are listed in (Supplementary Table 1). To validate the specificity of the primer to a target gene, PCR amplification was performed by modifying the protocol described by Aznar and Alarcon (2002). Briefly, PCR amplification was conducted using cDNA samples and Dream Taq Green PCR Master Mix (Thermo

scientific). The PCR cycling conditions were: an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, annealing temperature for 30 s, 72°C for 1 min, and a final 5 min extension at 72°C. Reactions were performed in a MyCyclerTM Thermal Cycler System (Bio-rad, Hercules, CA). The RT-PCR amplifications were carried out in 20 μL PCR reaction mixture containing diluted 1 μL of cDNA (100 ng), 10 μL AMPIGENE qPCR Green Mix Lo-ROX (Enzo life science, Farmingdale, NY), 1 μL of each forward and reverse primer (10 pmole) 7 μL of nuclease-free water. The RT-PCR was performed using a CFX connect real-time PCR Detection system (Bio-rad). The cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of amplification (95°C for 5 s and annealing temperature for 25 s). The relative quantification of gene expression was calculated after normalization to chicken glyceraldehyde-3-phosphate dehydrogenase and by using $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The GLM procedure of SAS was used to compare with egg quality in laying hens between SES and WES (SAS Institute, Cary, NC). The statistical significance was set at $P < 0.05$.

RESULTS

Comparison of Eggshell Quality Between Hens With SES or WES

The result for eggshell quality between SES and WES groups is presented in Table 1. Consistent with the screening model, SES group had a greater ($P < 0.01$) eggshell strength and thickness by 54.0% and 16.1% than WES group, respectively; however, no difference in eggshell colors was observed between groups.

Transcriptome Alignment and Mapping Statistics

After removing low-quality and adaptor sequences, a total of 136.48 million reads with an average of 17.06 million reads per sample (range: 14.54−19.09 million) were obtained. The GC contents of the libraries ranged from 47 to 50%, which were very close to 50%. The Q20 and Q30 quality values were 99.95% and 94.74%, respectively (Supplementary Table 2). The reads in each sample were mapped to the *Gallus gallus* 6.0 assembly of the chicken genome at average alignment rates of 96.29% and 96.70% for SES and WES groups, respectively.

Table 1. Egg quality of selected laying hens with either strong eggshell strength (SES) or weak eggshell strength (WES)[1].

Items	Group		SEM	*P*-value
	SES	WES		
Eggshell strength (kg/cm^2)	4.17	1.92	0.243	<0.01
Eggshell thickness (μm)	415	348	0.9	<0.01
Eggshell color (color fan)[2]	12.6	11.5	0.71	0.31

[1]Data are least squares means of 4 observations per treatment.
[2]Color fan scale; 15 = very dark brown and 1 = very light and pale.

DEGs Profiling

The DEG profiles were investigated between the SES and WES groups. The entire DEGs were identified 2,084 genes. Of the 2,084 genes, 1,358 genes were upregulated and 726 genes were downregulated in WES group compared with SES group (Figure 2A). The details of those DEGs are presented in Supplementary Table 3. The MDS plots of the DEGs were visualized for their distinct transcriptional profiles between SES and WES groups (Figure 2B).

GO Enrichment and KEGG Pathways Analysis for DEG

All DEGs were analyzed by GO enrichment and KEGG pathway enrichment analyses using the DAVID ver. 6.8 online tool to identify the differences in the hepatic physiological processes between SES and WES groups. A total of 107 GO terms were enriched (*P*-value < 0.1; Supplementary Table 4) and 45 GO terms were significant (*P*-value < 0.05, fold enrichment >2.0) as shown in Figure 3. The most significantly enriched GO terms included protein phosphorylation, canonical Wnt signaling pathway, activation of GTPase activity, regulation of vesicle fusion, epidermal growth factor receptor signaling pathway, positive regulation of osteoblast differentiation, protein processing, insulin receptor signaling pathway, and response to calcium ion in the BP category. In the CC category, external side of plasma membrane was enriched and was associated with processes such as membrane raft, exocyst, contractile fiber, and membrane attack complex. Protein kinase activity, which is related to 16 genes, was the most enriched term within MF category.

Moreover, the DEGs were enriched in 141 KEGG pathways (Supplementary Table 5) and the most significant pathways ($P < 0.05$; Figure 4) included mammalian target of rapamycin (**mTOR**) signaling pathway, Janus kinase (**JAK**)-signal transducer and activator of transcription (**STAT**) pathway, mitogen-activated protein kinase (**MAPK**) signaling pathway, insulin resistance (**IR**), and insulin signaling pathways. Additionally, the DEGs were predicted to play important roles in energy metabolism, particularly for fatty acid metabolism, and ascorbate and aldarate metabolism based on the KEGG pathway enrichment analysis.

Identification of Hub Genes and Pathways Through PPI Network Analysis of DEG

The PPI network was constructed including 418 nodes and 674 edges (Figure 5A). Based on the STRING database, the top 10 DEGs were evaluated in the PPI network using 5 centrality methods (Table 2). Furthermore, we identified the intersections of these 5 centrality

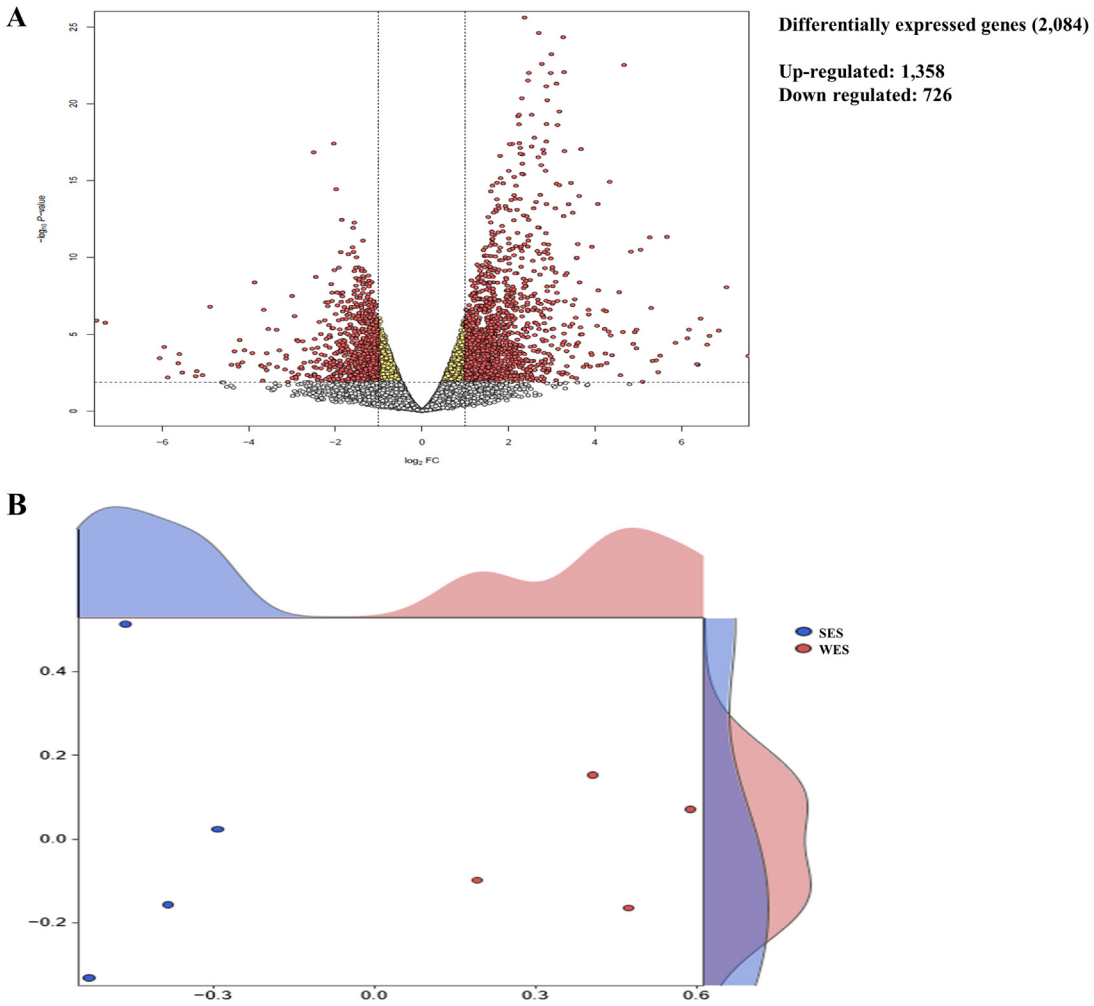

Figure 2. (A) Volcano plot of differently expressed genes (DEGs). The volcano plots illustrate the size and significance of the DEGs between strong eggshell strength (SES) and weak eggshell strength (WES) groups. The volcano plot reports -log (*P*-value) for genes as Y variables plotted against their respective $\log_2$ (fold change) as X variables. (B) The multidimensional scaling (MDS) plot showing the expression level of genes in 8 different samples. Blue spot represents SES groups and red spot represents WES groups.

methods and created a Venn plot (Figure 5B) to recognize significant hub genes using an online website (http://jvenn.toulouse.inra.fr/app/example.html). Ultimately, the 3 hub genes, including growth factor receptor bound protein 2 (***GRB2***), transferrin/ovotransferrin (***TF***), and Cbl proto-oncogene (***CBL***) were confirmed. The hub gene extracted from 5 centrality methods may correspond to key candidate genes with important regulatory functions to make a different intensity of eggshell strength of aged laying hens.

Furthermore, 3 significant modules, including module 1 (MCODE score = 10.6), module 2 (MCODE score = 8), and module 3 (MCODE score = 7) were created from the PPI network through the cut-off DEGs by MCODE (Figure 6). Genes of each module were investigated by biological functional enrichment analysis using DAVID database. Module 1 (Figure 6A), including 11 nodes and 53 edges, were remarkably enriched in 'endocytosis, 'ErbB signaling pathway', 'Jak-STAT signaling pathway', and 'insulin signaling pathway'. Moreover, module 2 (Figure 6B), including 8 nodes and 28 edges, were involved in 'ubiquitin mediated proteolysis, 'endocytosis, and 'calcium signaling pathway'. Furthermore, module 3 (Figure 6C) contains 15 nodes and 49 edges that are primarily related to 'calcium signaling pathway, 'regulation of actin cytoskeleton', and 'neuroactive ligand-receptor interaction' (Table 3).

Validation of DEG in the RNA-Sequencing Results

To validate RNA-seq expression results, 9 DEGs (Top 9 DEGs; 5 upregulated and 4 downregulated genes) were selected for qRT-PCR, which possessed the characteristics of abundant expression and higher FC. These genes were acid phosphatase 3 (***ACP3***), regenerating family member 1 alpha (***REG1A***), rhodopsin (***RHO***), adenylate cyclase 2 (***ADCY2***), transmembrane protein 86A (***TMEM86A***), regulator of G-protein signaling 16 (***RGS16***), angiotensin I converting enzyme (***ACE***), transient receptor potential cation channel subfamily M member 8 (***TRPM8***), and solute carrier family 7 member 11 (***SLC7A11***). The samples of qPT-PCR were same as those used for RNA-seq. The results confirmed

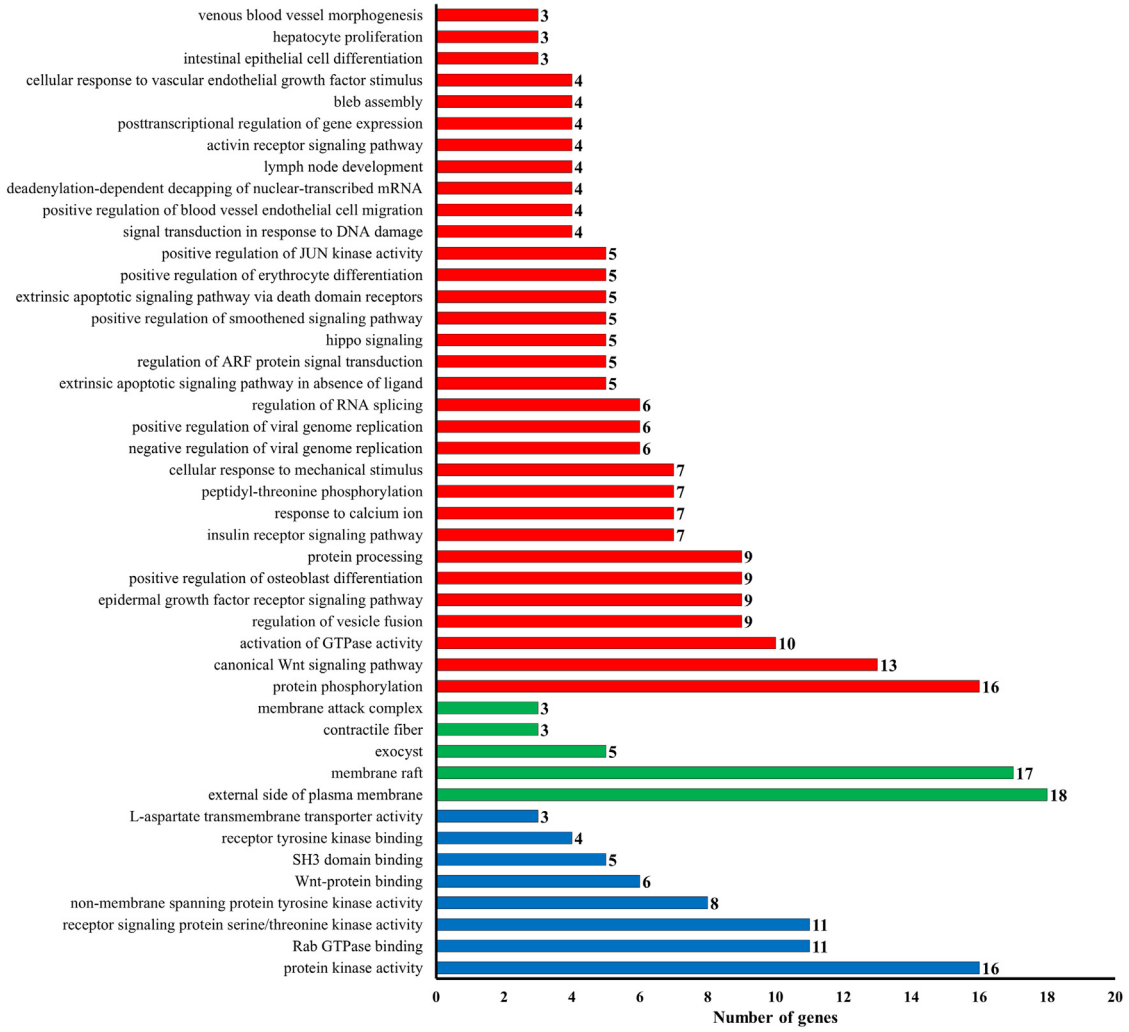

Figure 3. Most significantly enriched gene ontology (GO) terms associated with differentially expressed genes between different intensity of eggshell strength. GO categories are such as biological processes, cellular components, and molecular functions are shown in red, green, and blue color, respectively.

that the expression patterns of selected genes were consistent with those of RNA-seq (Figure 7).

DISCUSSION

Eggshell strength is one of the most important egg quality parameters because high incidence of soft or easily-cracking eggshells leads to a considerable economic loss in the layer industry (Stefanello et al., 2014). The main factors affecting eggshell strength have been associated with both animal (e.g., age, genetics, and health) and environmental factors (e.g., nutrition, temperature, management system; Roberts, 2004; Guo et al., 2015; Ketta and Tůmová, 2016). Therefore, many efforts have been made to improve eggshell strength by controlling those factors involved in eggshell strength of laying hens (Zhang et al., 2017; Qiu et al., 2020). Mineral nutrition has received the most attention, especially for Ca and P because nutritional management is convenient and practical in the layer industry and those specific minerals are the main components of eggshells (Ademosun and Kalango, 1973; Lim et al., 2003; Jiang et al., 2013b). It has been reported that increasing Ca concentrations with maintaining proper available P concentrations in diets improved eggshell strength of aged laying hens (Lim et al., 2003; An et al., 2016). In addition, Mg concentrations in diets was also reported to improve eggshell strength in laying hens (Seo et al., 2010; Kim et al., 2013a,b). However, the results from previous experiments have been inconsistent (Lim et al., 2003; Pelicia et al., 2009; An et al., 2016), such that no promising regimens have been identified. One possible reason for variable results is a lack of information for molecular mechanisms such as nutrient metabolism associated with eggshell quality in laying hens. Therefore, in the current study, we applied hepatic transcriptomic analysis to identify the role and regulation of the liver for eggshell strength using 2 distinct groups of laying hens with either strong eggshells or weak eggshells.

For the molecular analysis regarding eggshell strength in laying hens, Zhang et al. (2019) conducted a uterine transcriptomic analysis in laying hens with high or low eggshell strength during 3 different uterine calcification periods, and showed differences in extracellular proteins and ion transporters associated with eggshell

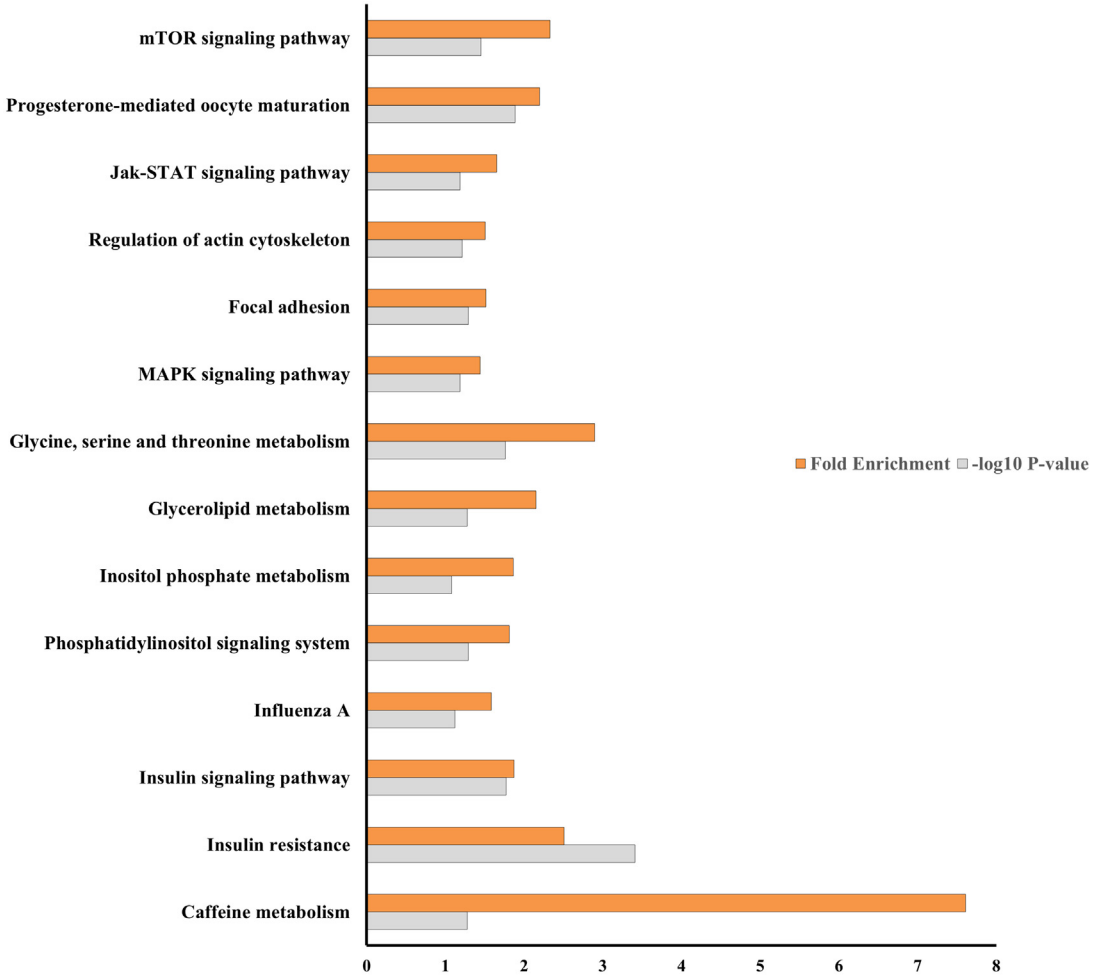

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG)-enriched pathways enriched with differentially expressed genes responsible for different eggshell strength were generated for liver tissue with -log10 *P*-values > 1.0

calcification. However, most of molecular studies related to eggshell strength have emphasized specifically the uterine function because eggshell formation occurs exclusively in the uterus (Brionne et al., 2014; Sah et al., 2018). Thus, few studies have analyzed other pivotal tissues that can potentially affect egg production and eggshell strength in laying hens.

The liver is the central metabolic organ associated with the metabolism of various nutrients, detoxification of exogenous substances, and host defense against invading pathogens (Crispe, 2009; Chiang, 2014). In addition, the liver plays a role in egg formation by participating in egg yolk synthesis and Ca transport (Galea, 2011). Therefore, the knowledge of the hepatic transcriptome might provide novel molecular functions of the liver, which may influence the eggshell strength in laying hens. However, a research pertaining to hepatic molecular functions on eggshells strength is largely lacking in laying hens.

As we intended, eggshell strength was significantly different between SES and WES groups (eggshell strength = 4.17 vs. 1.92 for SES and WES, respectively). Likewise, eggshell thickness for SES group was greater than for WES group. This result confirmed the high positive correlation between eggshell strength and thickness as reported previously (Zhang et al., 2005), which confirmed that the selected layer groups in the current study provided the reliable data for hepatic transcriptomic analysis of laying hens with different eggshell strength.

Based on the hepatic transcriptomic profile in the current experiment, liver homeostasis capacity (e.g., liver regeneration or fatty liver disease) is likely an important factor affecting eggshell strength of laying hens. In the current experiment, mTOR signaling pathway was enriched in DEGs between SES and WES group. The mTOR is a serine/threonine protein kinase that integrates various nutrient and hormonal signals to regulate cell growth and metabolism (Dann and Thomas, 2006). Bottje et al. (2014) reported that the mTOR pathway genes were upregulated in the liver of broiler chickens with high feed efficiency as compared to those with low feed efficiency, which indicates that mTOR pathway is highly involved in muscle development and protein metabolism. On the contrary, laying hens with low egg production had upregulated mTOR pathway-related genes in the pituitary gland than those with high egg production (Mishra et al. 2020). Moreover, previous poultry studies have reported that apoptosis caused by external stimulus (e.g., *Salmonella* infection or thermal stress) upregulated mTOR signaling (Arsenault et al., 2013; Napper et al., 2015).

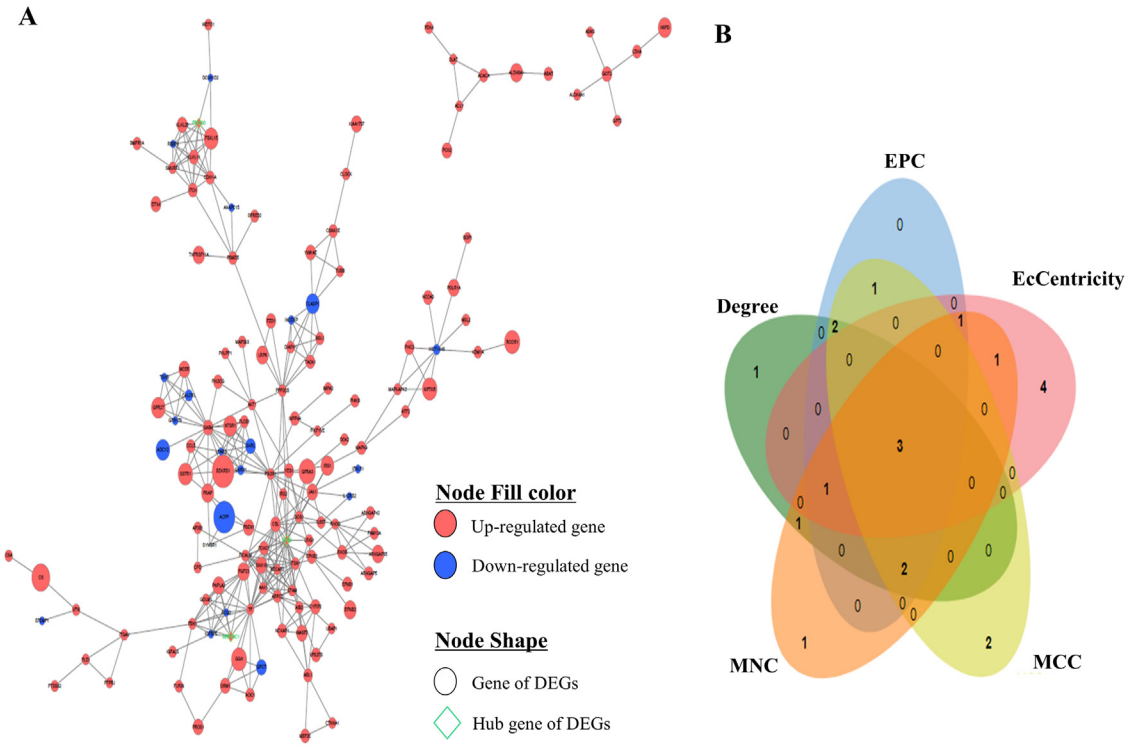

Figure 5. (A) Protein-protein interaction (PPI) network for the cutoff differentially expressed genes (DEGs) based on absolute $\log_2$ (fold change) of ≥1.5. A total of 418 nodes and 674 interaction associations were identified. The red node represents the upregulated gene, whereas the blue node represents the downregulated gene. The nodes with the highest degree scores were shaped as diamond and highlight the green border paint. Node size indicated the fold change of each gene. The node disconnected in the network remove. (B) A Venn plot to identify significant hub genes generated by five centrality calculation methods. The five centrality methods were Degree, EPC, EcCentricity, MCC, and MNC. Areas with different colors correspond to different algorithms. The cross areas indicate the commonly accumulated DEGs. The elements in concurrent areas are the 3 hub genes (*GRB2, TF*, and *CBL*).

Similar upregulation of genes related to mTOR signaling pathway in the liver was observed for WES group than SES group in the current experiment. The phosphatidylinositol-3 kinases (***PI3Ks***) family is involved in stimulating differentiation, proliferation, and survival of cells and participates in the signaling pathways related to the liver injury caused by fluorosis in the human body (Okkenhaug and Vanhaesebroeck, 2003; Fan et al., 2015). According to Zhu et al. (2017), phosphoinositide-3-kinase regulatory subunit 1 (***PIK3R1***) is a candidate gene for non-alcoholic fatty liver disease (**NAFLD**) in rats, indicating that the upregulation of this gene may represent the development of NAFLD. In the current experiment, *PIK3R1* was upregulated by a $\log_2$ FC of 1.62 in WES group than in SES group, indicating that decrease in eggshell strength is likely associated with the development of fatty liver disease in laying hens. Likewise, in another mTOR pathway-related gene, we also observed upregulation of ribosomal protein s6 kinase a3 and ribosomal protein s6 kinase a6 by a $\log_2$ FC of 1.33 and 1.16, respectively, in WES group. These genes encode a member of the ribosomal S6 kinase family of serine/threonine kinases that regulate diverse cellular processes such as cellular growth, motility, survival, and proliferation (Carriere et al., 2008). In addition, the expression of these genes in the liver was also reported to be increased in mice with fatty liver diseases (Wang et al., 2014; Simoes et al., 2020).

The present study also showed other evidence of alterations in gene expressions related to liver homeostasis

Table 2. Top 10 genes evaluated in the protein-protein interaction network between strong eggshell strength (SES) or weak eggshell strength (WES).

Degree		EPC		EcCentricity		MCC		MNC	
Gene	Score	Gene	Score	Gene	Score	Gene	Score	Gene	Score
GRB2	24	*GRB2*	74.024	*GRB2*	0.0328882	*TF*	408270	*GRB2*	23
TF	21	*CBL*	74.024	*FCHO1*	0.0281899	*GRB2*	403441	*CBL*	18
PIK3R1	18	*TF*	73.936	*SOS1*	0.0281899	*CBL*	403292	*TF*	17
CBL	18	*ARPC2*	73.893	*WASF2*	0.0281899	*STAM*	403208	*PIK3R1*	16
GNB4	18	*ITSN1*	73.847	*CYFIP2*	0.0281899	*ITSN1*	403202	*ARPC2*	13
STAM	13	*NECAP2*	73.718	*FGF23*	0.0281899	*PICALM*	403202	*GNB4*	12
ARPC2	13	*PIK3R1*	73.704	*PIK3R1*	0.0281899	*AAK1*	403200	*FGF23*	11
PPP2CB	12	*STAM*	73.655	*TF*	0.0281899	*NECAP2*	403200	*STAM*	11
ITSN1	12	*PICALM*	73.461	*YES1*	0.0281899	*ARPC2*	363000	*SOS1*	10
PICALM	12	*SOS1*	73.442	*CBL*	0.0281899	*SNX18*	362880	*BDKRB1*	10

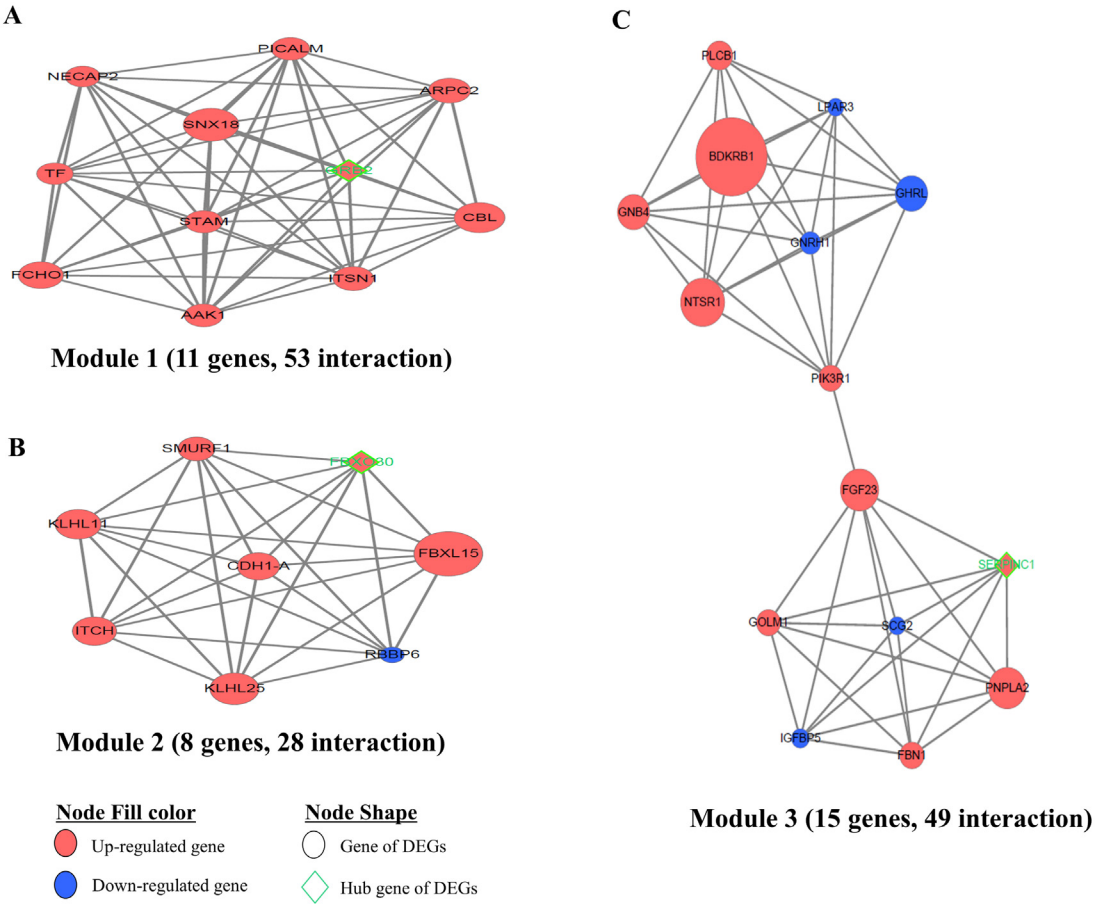

Figure 6. The three protein-protein interaction (PPI) hub network modules. The three significant modules, including (A) module 1 [molecular complex detection (MCODE) score = 10.6)], (B) module 2 (MCODE score = 8), and (C) module 3 (MCODE score = 7), were constructed from PPI network of differentially expressed genes using MCODE. The red node represents the upregulated gene, while the blue node represents the downregulated gene. The seed node of each module was shaped as diamond and highlight the green gene symbol. Node size indicated the fold change of each gene.

Table 3. The biological function enrichment analysis of the three protein-protein interaction (PPI) hub network modules.

Module	Database[1]	GO term/pathway	*P*-value	Genes[2]
module 1	KEGG	Endocytosis	0.0074	***ARPC2, STAM, CBL***
		ErbB signaling pathway	0.0525	***GRB2, CBL***
		Jak-STAT signaling pathway	0.0777	***GRB2, STAM***
		Insulin signaling pathway	0.0777	***GRB2, CBL***
	GO_MF	Protein binding	0.0351	***TF, GRB2***
module 2	KEGG	Ubiquitin mediated proteolysis	0.0284	***ITCH, SMURF1***
		Endocytosis	0.0506	***ITCH, SMURF1***
	GO_BP	Protein ubiquitination involved in ubiquitin-dependent protein catabolic process	0.0000	***ITCH, KLHL25, SMURF1, KLHL11***
		Proteasome-mediated ubiquitin-dependent protein catabolic process	0.0351	***SMURF1, KLHL11***
	GO_CC	Cytoplasm	0.0015	***ITCH, KLHL25, SMURF1, KLHL11, FBXL15***
		Calcium signaling pathway	0.0123	***KLHL11, FBXL15***
	GO_MF	Ubiquitin-protein transferase activity	0.0011	***ITCH, SMURF1, FBXL15***
		Ligase activity	0.0223	***ITCH, SMURF1***
		Ubiquitin protein ligase activity	0.0483	***SMURF1, FBXO30***
module 3	KEGG	Calcium signaling pathway	0.0256	***BDKRB1, PLCB1, NTSR1***
		Regulation of actin cytoskeleton	0.0306	***BDKRB1, PIK3R1, FGF23***
		Neuroactive ligand-receptor interaction	0.0627	***BDKRB1***, *LPAR3*, ***NTSR1***
	GO_BP	Negative regulation of apoptotic process	0.0187	*GHRL*, ***PIK3R1, NTSR1***
		Negative regulation of osteoblast differentiation	0.0355	*IGFBP5*, ***FGF23***
		Activation of MAPK activity	0.0483	*LPAR3, GHRL*
	GO_CC	Extracellular space	0.0039	*IGFBP5*, ***GOLM1***, *GHRL*, ***FGF23, FBN1***
		Axon	0.0058	*GNRH1, LPAR3, GHRL*

[1]Abbreviations: BP, biological process; CC, cellular component; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; MF, molecular function.

[2]Upregulated genes in WES groups are highlighted in bold and downregulated genes in normal typeface.

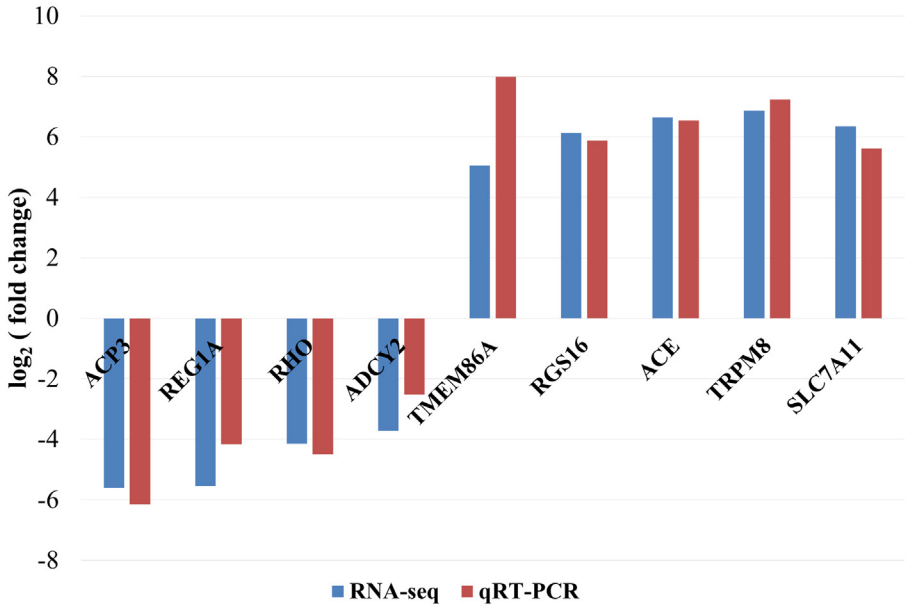

Figure 7. Illustrating of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) validation for selected differentially expressed genes in the liver tissue. The selected genes were placed in X-axis and Y-axis represents $\log_2$ (fold change) from qRT-PCR and RNA-sequencing.

capacity or fatty liver disease: Jak-STAT signaling pathway, MAPK signaling pathway, and IR pathway. The Jak-STAT signaling pathway plays a crucial role in signaling of cytokine receptors, various cell functions such as differentiation, proliferation, and apoptosis (Kisseleva et al., 2002; Truong et al., 2017). A previous study reported that Jak-STAT signaling pathway was significantly enriched in the liver of broiler breeder hens with low egg production induced by overfeeding as compared to restricted feeding (Wei et al., 2019). Moreover, it was reported that Jak-STAT signaling pathway was downregulated in the blood sample of laying hens fed with a dried olive pomace, indicating that Jak-STAT signaling pathway is also correlated with amelioration of immune responses (Iannaccone et al., 2019). This pathway was also highlighted in the adipose tissue of an experiment comparing with fatty liver hemorrhagic syndrome (**FLHS**) chickens and normal chickens (Zhu et al., 2021). In the current study, similar upregulation of genes related to Jak-STAT signaling pathway in the liver were observed for WES group compared with SES group. Interleukin (**IL**)-22 is a member of the IL-10 family of cytokines that act as mediators in inflammatory response and immune system in several tissues including the liver and pancreas (Khawar et al., 2016). The *IL-22* is mostly produced by dendritic cells, activated T cells, innate lymphoid cells (Khawar et al., 2016). The *IL-22* receptor subunit alpha 2 (***IL-22RA2***), which is known as *IL-22BP*, is an inhibitor of *IL-22* and is positively correlated with the severity of liver damage and fibrosis (Khawar et al., 2016; Weiskirchen and Tacke, 2016). In the present study, the expression of *IL-22RA2* was upregulated by a $\log_2$ FC of 2.25 in the WES group compared with SES group. Therefore, it is likely that WES group may suffer from liver damages compared with SES group. Moreover, in another Jak-STAT signaling pathway-enriched gene, AKT serine/threonine kinase 1 (***AKT1***) is a member of AKT responsible for the regulation of glucose uptake and plays a similar role to the PI3Ks family (Turinsky and Damrau-Abney, 1998; Fan et al., 2015). The expression of *AKT1* and PIK3 has been related to increase protection against liver injury, suggesting that a link between hepatocyte damage and impaired eggshell strength (Fan et al., 2015). Furthermore, PI3K and AKT signaling were upregulated in lipopolysaccharides-induced mice, demonstrating that these 2 genes may control oxidative stress-inducing inflammation (Zhong et al., 2016). In other words, upregulation of *IL-22* and *AKT1* observed in WES group compared with SES group may indicate that decreased eggshell strength may be linked to the liver damage or oxidative stress in laying hens. A recent study reported that laying hens induced oxidative stress by dietary arsenic supplementation had significantly lower eggshell strength, indicating that oxidative stress may be related to the deterioration of eggshell strength (Ma et al., 2021).

The MAPK signaling pathway is a regulator of hepatic metabolism (e.g., glucose metabolism and fatty acid metabolism) and participates in the modulation of inflammation response (Lawan et al., 2015; Shen et al., 2019). Luan et al. (2014) reported that MAPK signaling pathway was differentially expressed in the goose ovarian tissue at 2 stages of the egg-laying cycle (ceased period vs. laying period). Most of previous studies investigating MAPK signaling pathway in the liver have proved that NAFLD is alleviated via inactivation of the MAPK signaling pathway (Zhang et al., 2016; Shen et al., 2019). In the MAPK signaling pathway, fibroblast growth factor 23 (***FGF-23***) plays a role in maintaining normal phosphate homeostasis (Perwad et al., 2005). According to He et al. (2018), *FGF-23*

regulates fat content and distribution and is associated with IR in the liver. Furthermore, the patients with NAFLD had significantly higher serum *FGF-23* levels as compared to those without NAFLD (He et al., 2018). In the eggshell quality-related study, *FGF-23* signaling was involved in vitamin D metabolism and correlated with eggshell calcification, indicating that blocking the *FGF-23* signaling pathway increased eggshell strength of laying hens (Ren et al., 2018). In our results, *FGF-23* gene was also upregulated by a $\log_2$ FC of 2.73 in WES group compared with SES group. In another MAPK signaling pathway-enriched gene, serine/threonine-protein kinase 4 (***STK4***) is a stress-induced kinase and is correlated with apoptosis (Ready et al., 2017). It was reported that *STK4* was highly expressed in hematopoietic cells to promote the naive T lymphocyte proliferation and survival (Li et al., 2015b). In the present study, *STK4* expression in the liver was upregulated by a $\log_2$ FC of 2.31 in laying hens with WES than those with SES. Therefore, it can be hypothesized that hepatic injury stimulating the immune response in the liver of WES group may occur, leading to an increase in *STK4* expression.

The IR refers to a physiological condition in which the body's response to insulin is reduced below the normal standard response (Bugianesi et al., 2005). It is intimately related to NAFLD (Bugianesi et al., 2005; Watt et al., 2019). Pathological observations have shown a high level of IR in the FLHS model, which is known to be similar to NAFLD (Zhuang et al., 2019). Among the upregulated DEGs in IR pathway, Insulin receptor substrate proteins 1 (***IRS1***) is an important target of the insulin receptor tyrosine kinase (Copps and White, 2012). The *IRS1* signaling is required for glucose homeostasis and systemic growth in the liver (Dong et al., 2006). It appears that *IRS1* is a crucial regulator for the synthesis and oxidation of fatty acids in the liver of non-alcoholic steatohepatitis (**NASH**), which was significantly increased in the NASH rat model (Matsunami et al., 2011). Therefore, based on previous *IRS1* results, we speculate that high *IRS1* expression in WES group is closely related to FLHS. In another IR pathway-enriched gene, MAX-like protein X interacting protein (***MLXIP***), also known as *MondoA*, is responsible for glucose/fructose metabolism in human skeletal muscles and directs the transcription of genes in cellular metabolic pathways involved in diverse nutrient metabolism including fatty acid desaturation and elongation, triacylglyceride biosynthesis, glycogen storage (Ahn et al., 2019). Previous experiment indicated that mice-deficient *MondoA* have partially protected from IR and muscle fat accumulation (Ahn et al., 2019). In the current study, *MLXIP* expression was upregulated by a $\log_2$ FC of 2.64 in WES group compared with SES group. Therefore, we suggest that different intensity of eggshell strength in laying hens may be related to IR, and furthermore, laying hens with WES may show abnormal lipid metabolism such as fatty liver diseases.

Another functional pathway identified by KEGG analysis showed genes involved in nutrient metabolism, including the glycerolipid metabolism, and the glycine, serine, and threonine metabolism. Interestingly, all DEGs in both KEGG pathways were upregulated in WES group compared with SES group in this study. A previous uterine transcriptomic study regarding eggshell strength in laying hens showed similar results to our observation, indicating the glycerolipid metabolismin the liver may also influence on eggshell strength (Zhuang et al., 2019). Furthermore, both KEGG pathways were enriched in the hepatic transcriptome of broiler breeder hens fed diets supplemented with genistein, an isoflavone phytoestrogen known to have antioxidant effects and alleviate fibrosis of the liver (Lv et al., 2018).

In the glycerolipid metabolism pathway, cystathionine-beta-synthase like (***CBSL***) is known to be involved in catalyzing the conversion of serine and homocysteine to cystathionine and water (Jhee and Kruger, 2005). Homocysteine is proposed to modulate bone remodeling and strength (Zhou et al., 2019). A previous transcriptome study comparing the tibia from laying hens with good or poor bone quality showed that *CBSL* was highly expressed by 9-fold increase in laying hens with poor bone quality (De Koning et al., 2020). The bone-related disorders were reported to affect egg production and egg quality in laying hens because skeletal damage in laying hens leads to various problems with Ca metabolism associated with eggshell formation (Whitehead, 2004). In the present study, *CBSL* expression in the liver was upregulated by a $\log_2$ FC of 2.35 in laying hens with WES than those with SES. However, it remains unclear how the upregulation of *CBSL* in the liver affects poor bone quality. Further studies on *CBSL* functions in the liver of laying hens are necessary for elucidating its role in eggshell formation and strength.

The alignment of PPI networks allows a further investigation into the fundamental relationships among DEGs by associated genes and protein complexes with network pathways (Athanasios et al., 2017). The PPI network in the current study was created with DEGs, and then the top centrality hub genes were obtained using 5 centrality methods. Ultimately, we confirmed 3 hub genes, including *GRB2, TF*, and *CBL*, which were upregulated in WES group compared with SES group. *GRB2* contributes to the regulation of cellular functions, including proliferation and differentiation, as an important adaptor protein (Ge et al., 2017). A previous study suggested that downregulation of *GRB2* improved hepatic steatosis, glucose metabolism, oxidative stress, and apoptosis by modulating the insulin signaling pathway in HepG2 cells (Shan et al., 2013). According to Amanatidou and Dedoussis (2021), *GRB2* is one of the candidate NAFLD-related proteins. *TF* is one of the major matrix protein of hen eggshell membranes that consists of 12 to 13% of total egg white protein (Ko and Ahn, 2008). *TF* was reported to play a role as an acute phase protein in chickens and the level of *TF* in the blood was increased in response to inflammation (Rath et al., 2009). A comparison of eggshell matrix proteins between young and aged laying hens revealed that

aged laying hens had less eggshell strength than young laying hens and high concentrations of *TF* protein was observed in aged laying hens than in young laying hens (Panheleux et al., 2000). The expression of *TF* observed in this study is also coincident with the result from the previous study investigating the gene expression using integrated transcriptome and whole genome re-sequencing in the uterus of laying hens with different eggshell strength (Zhang et al., 2015). However, to our knowledge, no previous studies have revealed the mechanism involving *TF* expression and eggshell strength in poultry. The *CBL* is a protein coding gene, which functions as ubiquitin ligases and multifunctional adaptor molecules and plays role in the control of cell proliferation, differentiation and morphology, as well as in pathologies such as autoimmune diseases (Dikic et al., 2003). Overexpression of *CBL* is reported to reduce in vitro bone resorption (Miyazaki al., 2004). Our results also showed that the expression of *CBL* was upregulated in WES groups compared with SES groups. These findings lead us to hypothesize that differential expression of *CBL* impacts eggshell strength, which means that WES group may exhibit a lower ability of bone resorption than SES group. However, this hypothesis should be validated in further researches.

We composed 3 significant modules to further analyze the PPI network. In the present study, the genes of module 1 were upregulated in WES group and enriched in 'endocytosis', 'Jak-STAT signaling pathway', and 'insulin signaling pathway'. The seed node of module 1 is *GRB2,* which is one of the hub genes as mentioned above. Genes of module 2 enriched in 'ubiquitin mediated proteolysis' and 'calcium signaling pathway'. The seed node of module 2 is *FBXO30*, which acts as an ubiquitin ligase. A recent study indicated that expression of protein ubiquitination in goose fatty liver was higher than in the normal liver (Yan et al., 2020). This result appears to support other results regarding fatty liver development. Genes of module 3 were associated with 'calcium signaling pathway' and 'negative regulation of osteoblast differentiation. The seed node of module 3 is *SERPINC1*, one of the biomarkers for the prediction of NAFLD, showing upregulated in WES group (Pirola and Sookoian, 2018). Therefore, it is speculated that the pathways and genes associated with fatty liver and liver homeostasis in laying hens influence eggshell strength deterioration. However, there were no studies on the closed relationship between fatty liver diseases and the deterioration of eggshell strength. However, several previous studies in laying hen with fatty liver disorder found decreased egg production rate (Walzem et al., 1993; Jiang et al., 2013a) and increased eggs with a bad eggshell quality as compared to the control group (Galea, 2011).

In conclusion, we conducted a comparative transcriptomic analysis in the liver of aged laying hens displaying different eggshell strength. KEGG pathway analysis reveals that the relevant genes are enriched in the pathways related to fatty liver and liver homeostasis capacity. Genes related to fatty liver development are upregulated in laying hens with WES as compared to those with SES. Several genes associated with age-related alterations in oxidative stress and bone resorption are also identified in aged laying hens with WES. These findings of the current study provide some potential clues for understanding the molecular mechanism in the liver of aged laying hens with different intensity of eggshell strength, and therefore, serve as valuable reference data for future researches regarding improvements in eggshell strength of aged laying hens.

DISCLOSURES

The authors declare no conflict of interest for the data presented in this experiment.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102217.

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