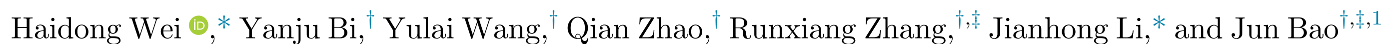
Serum bone remodeling parameters and transcriptome profiling reveal abnormal bone metabolism associated with keel bone fractures in laying hens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ABSTRACT Keel bone fractures affect welfare, health, and production performance in laying hens. A total of one hundred and twenty 35-wk-old Hy-line Brown laying hens with normal keel (**NK**) bone were housed in furnished cages and studied for ten weeks to investigate the underlying mechanism of keel bone fractures. At 45 wk of age, the keel bone state of birds was assessed by palpation and X-ray, and laying hens were recognized as NK and fractured keel (**FK**) birds according to the presence or absence of fractures in keel bone. The serum samples of 10 NK and 10 FK birds were collected to determine bone metabolism-related indexes and slaughtered to collect keel bones for RNA-sequencing (**RNA-seq**), Micro-CT, and histopathological staining analyses. The results showed that the concentrations of Ca, phosphorus, calcitonin, 25-hydroxyvitamin D_3, and osteocalcin and activities of alkaline phosphatase and tartrate-resistant acid phosphatase (**TRAP**) in serum samples of FK birds were lower than those of NK birds ($P < 0.05$), but the concentrations of parathyroid hormone, osteoprotegerin, and corticosterone in serum samples of FK birds were higher than those of NK birds ($P < 0.05$). TRAP staining displayed that FK bone increased the number of osteoclasts ($P < 0.05$). Micro-CT analysis indicated that FK bone decreased bone mineral density ($P < 0.05$). Transcriptome sequencing analysis of NK and FK bones identified 214 differentially expressed genes (**DEGs**) ($|\text{log2FoldChange}| > 1$, $P < 0.05$), among which 88 were upregulated and 126 downregulated. Kyoto Encyclopedia of Genes and Genomes pathway (**KEGG**) analysis indicated that 14 DEGs related to skeletal muscle movement and bone Ca transport (COL6A1, COL6A2, COL6A3, PDGFA, MYLK2, EGF, CAV3, ADRA1D, BDKRB1, CACNA1S, TNN, TNNC1, TNNC2, and RYR3) were enriched in focal adhesion and Ca signaling pathway, regulating bone quality. This study suggests that abnormal bone metabolism related to keel bone fractures is possibly responded to fracture healing in laying hens.

Key words: keel bone fracture, bone metabolism, bone health, RNA-seq, laying hen

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INTRODUCTION

Keel bone damage, including deviations and fractures, is a severe welfare and health issue in laying hens. In particular, keel bone fractures (**KBF**s) have been reported to alter behavior, affect physiology, and reduce production performance and egg quality of laying hens housed in alternative housing systems, such as enriched cages, aviary and cage-free systems (Hardin et al., 2019). The genetic background and age of birds, housing systems, nutrition, and perches affect the occurrence of KBFs (Hardin et al., 2019; Rufener and Makagon, 2020). There is a significant difference in the incidence of KBFs between brown- and white-feathered laying hens, and white birds had a higher incidence of KBFs than brown birds (Stratmann et al., 2015a). The incidence of KBFs in brown-feathered hens is higher than white-feathered hens (Candelotto et al., 2017). These inconsistent results are related to body weight and egg production of laying hens. In general, brown hens have heavier body weight and lower egg production than white hens. A recent study demonstrated that KBFs were strongly associated with egg production, and laying hens with a high egg laying rate had an elevated incidence of KBFs (Eusemann et al., 2020). In addition, studies found that the incidence of KBFs increased with the age of laying hens, and the highest KBFs occurred at the peak period of laying and gradually decreased after 45 weeks of age (Gebhardt-Henrich et al., 2015; Wei et al., 2020). Therefore,

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KBFs could be associated with egg production because higher egg laying rate requires more Ca for eggshell formation, which affects bone metabolism and quality in laying hens.

With the rapid development of genomics, RNA sequencing (**RNA-seq**) has become the most common method of genomics research at the transcriptional level and has been wisely applied to study growth and development, disease screening, genetic diversity, and skeletal development in poultry and other animals. In recent years, transcriptome sequence analysis has been used to explore growth traits, meat quality characteristics, regulation of production performance, and mechanism of nutrient digestion and absorption in chickens (Teng et al., 2019; Ma et al., 2021). A few studies have examined the relationship between bone growth, development and metabolism, and bone health in laying hens through genomics methods. Guo et al. (2017) and Raymond et al. (2018) identified several strong candidate genes related to the genetic architecture of bone quality variation in laying hens using a genome-wide association study, and these genes could regulate bone strength and bone mass, affecting bone fractures. A study investigated pathophysiological characteristics of bone in laying hens fed a low Ca diet through RNA-seq and identified some osteoporosis-related differentially expressed genes (**DEGs**), indicating that dietary Ca deficiency induced bone damage via modulating the abnormal expression of bone quality-related genes (Jiang et al., 2019). The above studies have demonstrated that transcriptome sequencing analysis is a reliable and effective method to identify candidate genes related to bone quality and health in chickens.

As an important structural bone, the keel plays a supporting and protective role in the flapping wings and respiration of birds. Keel bone damage, especially fracture, reduces egg production, eggshell quality, and welfare, causes physiological stress, alters behaviors, and induces negative emotions in laying hens (Candelotto et al., 2017; Wei et al., 2020). Thus, KBFs are a challenging health problem for laying hen production farming. At present, the potential solutions to reduce KBFs in laying hens include dietary omega-3 polyunsaturated fatty acids or 25-hydroxyvitamin D_3 (**25-OHD$_3$**) supplementation (Tarlton et al., 2013), providing soft perches or ramps between perches in cage and aviary systems (Stratmann et al., 2015a, b), providing exercise space and enriched environment during pullet rearing (Casey-Trott et al., 2017a, b) and optimizing perch positioning and enriched resources (Rufener et al., 2020; Norman et al., 2021).

Although several studies have focused on the solutions to reduce the risk of KBFs and explored its causative factors in laying hens, the underlying mechanism of bone fragility remains unclear. Therefore, this study aimed to identify some candidates related to keel bone quality of laying hens housed in furnished cages through RNA-seq analysis. Our study suggests that these candidate genes (DEGs) from RNA-seq related to bone structure and metabolism that affect keel bone health in laying hens by regulating their abnormal expression at the transcription level.

MATERIALS AND METHODS

Ethics Statement

All experiments were approved by and conducted according to the guidelines of the Institutional Animal Care and Use Committee of Northeast Agriculture University (NEAU-[2011]-9).

Animals and Experimental Design

A total of 120 healthy 35-wk-old Hy-line Brown laying hens with normal keel (**NK**) bone and similar body weight (2.11 ± 0.03 kg) were used in this study. All laying hens were housed in 12 furnished cages with 10 birds per page, and the experiment lasted for 10 weeks from 35 to 45 weeks of age. The size of each cage was 150 cm × 70 cm × 70 cm (length × width × height), and enriched with two wooden square perches, one elevated closed red nesting box, a piece of plastic mesh, one rectangular feeder, and one waterline with four nipple drinkers. The detailed information on the layout of these resources in the furnished cage was consistent with our previous study (Wei et al., 2022). These furnished cages were placed in a semi-enclosed hen house with natural ventilation. The hen house had a combination program of natural and artificial lights. Artificial light was provided daily for 16 h of light (5:30−21:30 h) and 8 h of darkness, and light intensity was 18 to 22 lux. The ambient temperature range of the hen house was 21°C to 24°C, and relative humidity was 50% to 65%. All birds were given free access to food and water during the entire experiment period from 35 to 45 wk of age. A commercial corn-soybean meal layer diet (Wellhope Animal Husbandry Co., Ltd., Harbin, China) with 2,800 kcal/kg metabolic energy and 16.08% crude protein was provided to feed laying hens.

Assessment of Keel Bone Fractures

At the start (35-wk-old) and the end (45-wk-old) of the experiment, the keel bone status of each laying hen was assessed using palpation and a portable X-ray machine (WAT-LES100D, Shenzhen, China) according to our previous report (Wei et al., 2022). Palpation mainly assessed whether keel bone deviation was present, while X-ray primarily checked whether keel bone fractures were present. Briefly, the palpation was performed by a worker on the spinal or ventral and lateral surfaces of keel bone by running the thumb and forefinger and feeling weather keel deformities were present, such as S-deviations, sharp bends, callus, and others. If a keel with S-deviations, bending, and bumps was recognized as NK bone, and the keel with sharp bends, fragmented sections, callus materials, and shearing was considered as FK bone (Casey-Trott et al., 2015). After the palpation, X-ray was used to verify the reliability of palpated results, and fractures were recognized as keel bone with thickened bone or with black lines in the image. In this study, laying hens were classified into NK

and fractured keel (**FK**) birds based on the absence or presence of fractures in each keel bone. A NK bone that means that it is neither deviated nor fractured. Based on the assessment result of keel bone fractures, there were 15 FK laying hens, 16 deviated keel bone laying hens, and 89 NK laying hens at 45 wk of age. These NK and FK laying hens were individually marked with different leg-tags for sample collection.

Sample Collection

After assessing keel bone fractures, 10 FK with similar locations and severity of fractures and 10 NK laying hens were selected as focal animals for collecting samples. First, the blood of these focal animals was separately collected from the wing vein by venipuncture and centrifugated at 3,000 rpm for 15 min at 4°C. Serum samples were stored at −20°C to determine bone metabolism-related indexes. Finally, all focal animals were slaughtered by cervical dislocation, and keel bone was excised from the body. Muscle and other soft tissues attached to the surface of keel bone were removed by scissors and scalpel, and keel bone samples were fast-frozen in liquid nitrogen and stored at −80°C for bone mineral density (**BMD**, n = 3 each group), histopathological staining (n = 3 each group), and RNA-seq analyses (n = 4 each group). These assays were performed on different keel samples. The fracture is mainly located in the caudal part of each keel bone, and the visual pictures of NK and FK bones are shown in Figure 1.

Measurement of Serum Bone Metabolism-Related Indexes

The indexes related to Ca and P metabolism and bone remodeling in serum samples of NK and FK laying hens were measured. The concentrations of serum Ca and P were measured by Ca and P detection kit assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of serum 25-OHD$_3$, 1,25-hydroxyvitamin D$_3$ (**1,25-(OH)$_2$D$_3$**), calcitonin (**CT**) (Shanghai Jinma Laboratory Equipment Corporation Ltd., Shanghai, China), and parathyroid hormone (**PTH**, Shanghai enzyme-linked Biotechnology Corporation Ltd., Shanghai, China) were measured by the enzyme-linked immunosorbent assay (**ELISA**) method according to the kit's instructions.

The levels of bone remodeling-related indexes include osteocalcin (**OC**), alkaline phosphatase (**ALP**), tartrate-resistant acid phosphatase (**TRAP**), and osteoprotegerin (**OPG**) in serum samples of NK and FK laying hens were measured by the corresponding ELISA kits (Shanghai Jinma Laboratory Equipment Corporation Ltd., Shanghai, China) following the kit's instructions. The activity of serum corticosterone (**CORT**) was detected with an ELISA kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 50 μL of serum sample containing 10 μL of serum and 40 μL of sample dilution from per focal animal was added into the micropore to measure the level of each physiological index. The measurement of each sample was performed in triplicate for each index, and their mean value was calculated as a final value. Optical density (**OD**) values were then measured at the corresponding wavelength of each index using a microplate reader (Biotek Instrument Inc., Winooski, VT). Finally, the level of each sample per index was calculated by the corresponding standard curve and OD values.

Keel Bone TRAP Staining

A piece of 0.5 cm long keel sample at fracture position of FK bone and a piece located at a similar position in NK bone were cut, and the transverse plane of the cut piece was subjected to histopathological observation according to previous report (Wei et al., 2021a). The cut keel bone samples were first fixed with 4% paraformaldehyde for one week and then decalcified with 10%

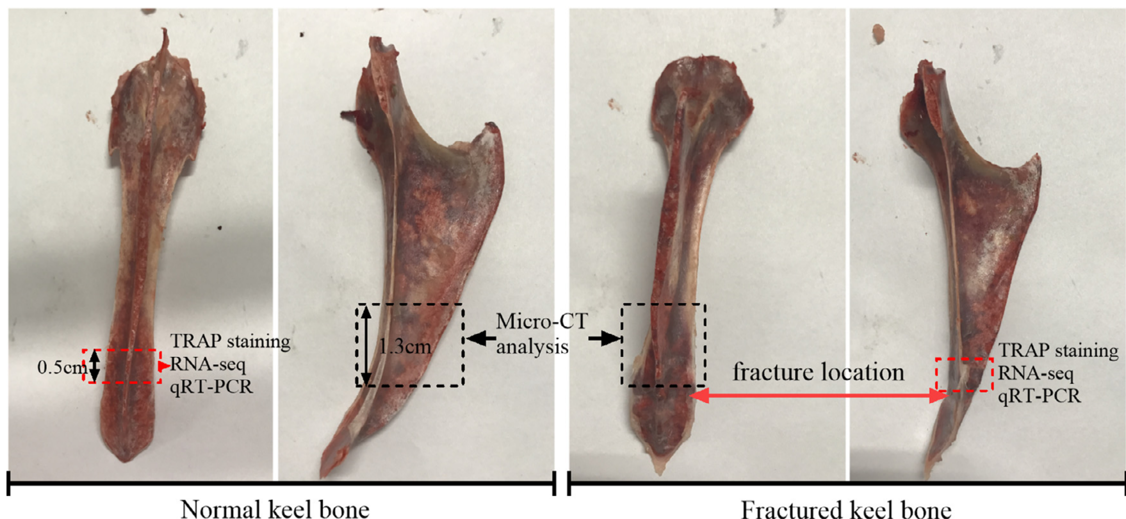

Figure 1. Visual images of normal keel (NK) and fractured keel (FK) bones used in this study. The red and black dotted rectangle represents the sampling location for different assays.

ethylene diamine tetraacetic acid for 5 min. The bone sample was embedded into the paraffin and sliced at a thickness of 5 μm. The paraffin-embedded sections of NK and FK bone samples were dewaxed with xylene solution and ethyl alcohol and incubated in distilled water. The sections were hatched using the filtered TRAP staining solution and counterstained with hematoxylin. The sections were dehydrated with xylene and sealed with neutral balsam, and the sealed sections were observed and examined using an orthostatic light microscope (Nikon Eclipse E100, Nikon, Tokyo, Japan). Finally, the image was taken under a 400 × optical microscope, and the number of osteoclasts was counted using image analysis software for microscope (Image-Pro Plus 6.0, Media Cybernetics, MD).

Keel BMD Measurement by Micro-CT

About a 1.3 cm long keel sample from fracture position towards keel cranial side in FK bone and the corresponding location in NK bone were cut and the transverse plane of the cut samples was used to measure keel BMD. The keel bone samples were soaked in 4% paraformaldehyde solution for one week and used to measure BMD by a Micro-CT scanner (μCT 50; Scanco Medical AG., Zürich, Switzerland). The condition of the Micro-CT scanner was set at 16 μm voxel size, 70 kV source potential, 200 μA current, 300 ms integration time, and 0.5 mm aluminum filter. After the scanning, screened image was restructured by software Mimics 19.0. The threshold was set at 200 mg HA/ccm (grayscale) for keel bone samples. Finally, three-dimensional model was analyzed by the Magics 19.01 and advanced bone analysis software (Healthcare Locus SP, Connecticut, USA) to determine keel BMD.

Total RNA Extraction and Complementary DNA (cDNA) Library Construction

Total RNA was extracted from 4 NK and 4 FK bone (fractured part) samples using TRIzol reagent (Invitrogen, Carlsbad, CA), and the concentration, purity, and integrity of total RNA were determined by ultraviolet spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA) and agarose gel electrophoresis. cDNA library was constructed following the instructions of the Illumina Kit (TruSeq RNA Sample Preparation Kit V2, Illumina, San Diego, CA). Before sequencing analysis, messenger RNA (**mRNA**) was purified from total RNA by magnetic beads containing poly A oligos, and mRNA was fragmented by ion interruption to form short fragments of 200 to 300 bp. First-strand cDNA was synthesized by random oligonucleotides and SuperScript II using these fragments as templates. Second-strand cDNA was synthesized using DNA polymerase I and RNase H. A paired-end library was built using synthesized cDNA by a Genomic Sample Prep Kit (Illumina, San Diego, CA). To get cDNA fragments (380 bp), library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA), and DNA fragments with ligated adaptor molecules on both ends were enriched using Illumina PCR Primer Cocktail in a 15-cycle polymerase chain reaction (**PCR**). Products were purified by the AMPure XP system and quantified using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent, Santa Clara, CA). The sequencing library was sequenced on a NovaSeq 6000 platform (Illumina, San Diego, CA) by Shanghai Personal Biotechnology Cp. Ltd., Shanghai, China.

Transcriptome Sequencing and Bioinformatics Analysis

The raw reads from RNA-seq were filtered using Cutadapt software, and the reads with adaptor contamination and low quality were removed. The remaining (clean) reads were mapped to the *Gallus* genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000002315.3_Gallus_gallus4.0/GCF_000002315.3_Gallus_gallus-4.0_genomic.gff.gz) using HISAT2 software (http://ccb.jhu.edu/software/hisat2/index.shtml). In the process of mapping, mismatches of no more than two bases were allowed. Transcript expression of genes was normalized by fragments per kilobase of exon model per million mapped reads (**FPKM**). DEGs between groups were identified based on genes with $|\log2(\text{FoldChange})| > 1$ and $P < 0.05$ using the DEGseq R package. The functional enrichment analysis of DEGs was performed by the Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**). GO database was used for GO term analysis, and the KEGG database was used for pathway analysis and functional annotation of DEGs.

Real-time Quantitative PCR (qRT-PCR) Validation of DEGs

To validate the accuracy of RNA-seq results, a total of 14 DEGs enriched in focal adhesion (collagen type VI alpha 2 chain (**COL6A2**), tenascin N (**TNN**), platelet-derived growth factor subunit A (**PDGFA**), collagen type VI alpha 3 chain (**COL6A3**), collagen type VI alpha 1 chain (**COL6A1**), myosin light chain kinase 2 (**MYLK2**), epidermal growth factor (**EGF**), and caveolin 3 (**CAV3**)) and Ca signaling pathway (adrenoceptor alpha 1D (**ADRA1D**), bradykinin receptor B1 (**BDKRB1**), myosin light chain kinase 2 (**MYLK2**), troponin C2 (**TNNC2**), troponin C1 (**TNNC1**), calcium voltage-gated channel subunit alpha1 S (**CACNA1S**), and ryanodine receptor 3 (**RYR3**)) related to skeletal muscle movement and bone Ca transport were selected and validated by qRT-PCR. The special primer sequences of target genes are listed in Table 1. Total RNA was extracted from NK and FK bones using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase and PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). cDNA of each sample was five-fold diluted with sterile water and

Table 1. Specific primer sequences of target genes for qRT-PCR in this study.

Gene	Reference number	Primer sequence (5'→3')
18S	AF173612	Forward: CGAAAGCATTTGCCAAGAAT Reverse: GGCATCGTTTATGGTCGG
CACNA1S	NM_001305147.1	Forward: CCGCCATCACCTTCCTCATTCAAG Reverse: TCTCCACTTCGTCCATCTCCATCTG
TNN	XM_040677518.1	Forward: GCTGGAAGGACTGGAACAAGGAAC Reverse: CCGCAGGGTATGGGTAGAGGAAG
TNNC1	NM_205133.1	Forward: TGCCTTCGACATCTTCGTGCTG Reverse: GCCATCCTCATCCACCTCATCAATC
TNNC2	NM_205450.2	Forward: GGGACATCAGCACCAAGGAGTTG Reverse: CGCACCATCATCACCAGGAACTC
MYLK2	NM_205392.1	Forward: GAACCACCGCAACCTCATCCAG Reverse: CGAACACCATGCAGTCCACCTC
RYR3	NM_206874.2	Forward: GCAAGAAGCCATACGGATCTCAGAG Reverse: AATCTCCTCCTCCTCCTCCTCCTC
ADRA1D	MK138998.1	Forward: GGTCATCCTGGTGGTGGTCTGG Reverse: TGGCTCCTCGGTGATGCTACAG
BDKRB1	NM_001080720.1	Forward: TTCCCTTCTCAAACCAGACCAACAC Reverse: ACACAGATGGCGTCGATACACTTG
CAV3	NM_204370.2	Forward: CACCACCTTCACCGTCAGCAAG Reverse: AAGGAGATGAGGGCGAAGAGGAAG
EGF	NM_001001292.1	Forward: GCCGCCTATTGCTCCAGAAGTC Reverse: CACTGCTACACTCCTTGACAACCG
PDGFA	NM_204306.1	Forward: TGGCTACCTGTCCTTTACCTCCTG Reverse: GGATGCTGTGGATCTCGCTGTG
COL6A1	NM_205107.1	Forward: GTACTTCCGCTGTGACCGCTTC Reverse: TGATGGCTGACACGCTGTTCTTC
COL6A2	NM_205348.3	Forward: TCTTCCCACTGACCTACAACCTGAC Reverse: TTGATGGCGTGTATGATGGCTGAC
COL6A3	NM_205534.2	Forward: CAAGCGGCAGGAGAAGACAAGG Reverse: GAGTGGTCGTGTGCTAAGGTGATG

stored at −80°C. qRT-PCR was performed on Light Cycler 96 qPCR system (Roche, Rotkreuz, Switzerland). The reaction mixture of qRT-PCR contained 10 μL, including 5 μL of 2X Roche Fast Universal SYBR Green Master kit (Roche, Mannheim, Germany), 3.4 μL of PCR water, 1 μL of diluted cDNA sample, 0.3 μL of forward and reverse primers. The conditions of qRT-PCR were set as follows: 95°C for 10 min, 40 cycles of 95°C for 15s, and 60°C for 1 min. 18S was used as an internal reference gene, and then $2^{-\Delta\Delta Ct}$ method was applied to quantify the relative expression of DEGs.

Statistical Analysis

All data were analyzed by software SPSS 22.0 (SPSS Inc., Chicago, IL). Before analysis, the data were tested for normality by a Kolmogorov−Smirnov test, and an independent sample t-test was performed to compare the differences in serum Ca-P metabolism and bone remodeling indexes, bone mineral density, and validated results of DEGs between NK and FK laying hens. The results were expressed as mean ± standard error of the mean (**SEM**), and the difference was considered statistically significant at $P < 0.05$.

RESULTS

Determination of Serum Ca and P Metabolism-Related Indexes

The results of serum Ca and P metabolism-related indexes in laying hens are shown in Table 2. Compared to NK laying hens, the concentrations of Ca, P, CT, and 25-OHD$_3$ were significantly increased ($P < 0.05$), and those of PTH were significantly decreased ($P < 0.05$) in serum samples of FK laying hens. However, there was no significant difference in the concentration of serum 1,25-(OH)$_2$D$_3$ between NK and FK laying hens ($P > 0.05$).

Determination of Serum Bone Remodeling-Related Indexes

The results of serum osteoblasts and osteoclasts activity-related indexes in laying hens are shown in Table 3. The activities of serum ALP and TRAP and concentration of serum OC in FK laying hens were significantly lower than those in NK laying hens ($P < 0.01$). However, the levels of OPG and CORT in serum samples of FK laying hens were significantly higher than those in NK laying hens ($P < 0.01$).

Table 2. Determination of serum Ca and P metabolism-related indexes in NK and FK laying hens.

Index (unit)	NK group	FK group	P-value
Ca (mmol/L)	1.99 ± 0.12	1.64 ± 0.04	0.020
P (mmol/L)	2.16 ± 0.07	1.89 ± 0.08	0.025
1,25-(OH)$_2$D$_3$ (pg/mL)	113.94 ± 1.25	112.82 ± 2.06	0.649
25-OHD$_3$ (ng/mL)	7.57 ± 0.07	7.13 ± 0.15	0.026
PTH (ng/L)	37.40 ± 0.25	42.34 ± 0.17	< 0.001
CT (ng/L)	85.11 ± 0.73	80.89 ± 1.06	0.005

Abbreviations: Ca, calcium; CT, calcitonin; FK, fractured keel bone; NK, normal keel bone; P, phosphorus; 1,25-(OH)$_2$D$_3$, 1,25-hydroxyvitamin D$_3$; 25-OHD$_3$, 25-hydroxyvitamin D$_3$; PTH, parathyroid hormone.

Table 3. Determination of serum bone remodeling-related indexes in laying hens.

Index (unit)	NK group	FK group	*P*-value
ALP (U/L)	2111.08 ± 27.78	1872.68 ± 19.35	< 0.001
TRAP (U/L)	185.29 ± 1.89	160.48 ± 1.31	< 0.001
OPG (ng/L)	460.74 ± 8.96	512.01 ± 4.16	< 0.001
OC (ng/L)	8.61 ± 0.04	8.11 ± 0.08	< 0.001
CORT (ng/L)	385.12 ± 3.02	404.57 ± 4.91	0.005

Abbreviations: ALP, alkaline phosphatase; CORT, corticosterone; FK, fractured keel bone; NK, normal keel bone; OC, osteocalcin; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase.

Bone Histopathological Examination

The results of the keel bone histopathological examination are shown in Figure 2. Figure 2A represents TRAP staining of keel bone samples, and the results showed that FK bone had significantly elevated ($P < 0.05$) number of osteoclasts than NK bone (Figure 2B).

Determination of Keel BMD

The results of keel BMD measurement by Micro-CT are presented in Figure 3. Figure 3A represents the analyzed region of interest in NK and FK bones using a Micro-CT scanner. As shown in Figure 3B, the BMD of FK bone was significantly lower than NK bone ($P < 0.05$).

Transcriptome Data

The results of RNA-seq and mapping of NK and FK bones are displayed in Table 4. The raw and clean reads of each library were more than 36 million and 33 million, respectively, and the average rate of clean reads was beyond 93.13%. The clean reads were mapped to the *Gallus* genome with a mapping rate ranging from 91.32% to 92.59%. The average percentage of Q30 bases was 92.34% in all samples. Figure 4A represents the result of correlation analysis among samples, which showed that the correlation coefficient was above 0.92, indicating the reliability of the mapping data. Figure 4B represents a principal component analysis (**PCA**) result of samples between groups, which displayed that inter-group samples had a significant difference in the principal components.

Identification of DEGs

A total of 214 DEGs were identified (|log2Fold Change| > 1, $P < 0.05$) in NK and FK bones by DESeq analysis, which included 88 upregulated and 126 downregulated DEGs (Figure 5A, Table S1). The clustering analysis of DEGs showed that eight samples from two groups had obvious clustering and high and low expression gene distribution between groups (Figure 5B).

GO and KEGG Pathway Enrichment Analyses of DEGs

To analyze the function of DEGs, GO and KEGG pathway enrichment analyses were performed. In this study, 214 DEGs were enriched in 20 GO terms consisting of 11 cellular components (**CC**), 7 biological processes (**BP**), and 2 molecular functions (**MF**) (Figure 6A). According

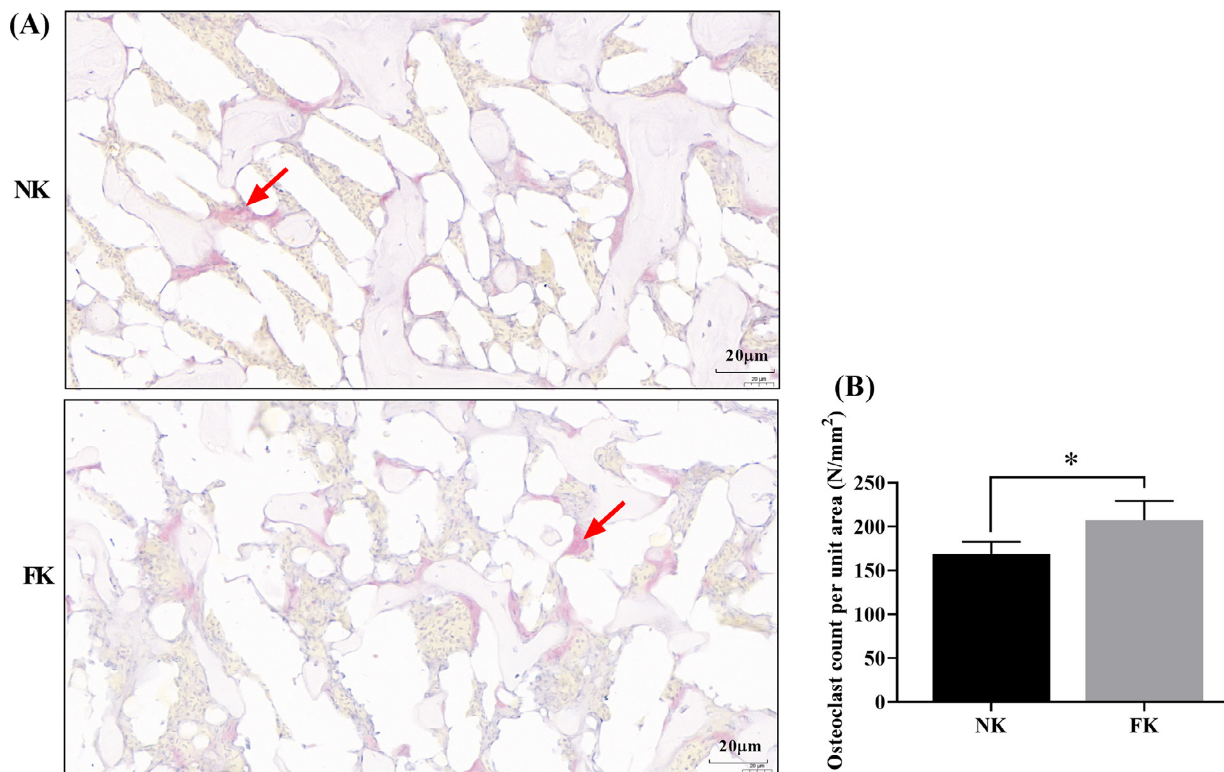

Figure 2. Results of tartrate-resistant acid phosphatase (TRAP) staining of normal keel (NK) and fractured keel (FK) bones in laying hens. (A) The images of NK and FK bone TRAP staining, and (B) the number of osteoclasts in NK and FK bones. Red arrow points to osteoclasts.

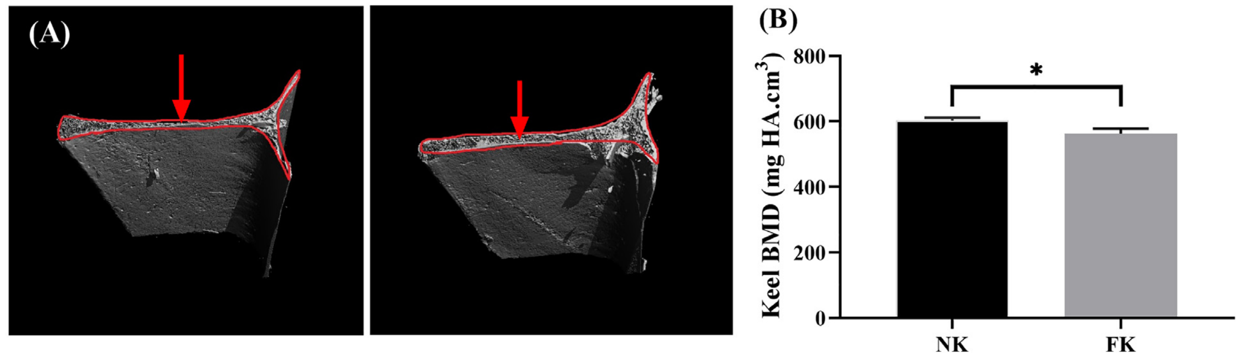

Figure 3. Analysis of BMD of normal and fractured keel bones by Micro-CT. (A) The area surrounded by red line represents the scanned region (transverse plane) of cut keel bone samples for Micro-CT analysis, and (B) BMD difference in normal (NK) and fractured keel (FK) bones in laying hens.

to the abundance of DEGs-enriched GO terms, the most abundant GO terms were in myofibril, contractile fiber, sarcomere, and contractile fiber part of cellular components and skeletal muscle contraction of biological processes, in which there were 19, 19, 17, 17, and 7 DEGs-involved, respectively. Additionally, 214 DEGs were enriched in 53 KEGG pathways in this study. The top 20 of KEGG pathways are displayed in Figure 6B. Of these pathways, DEGs were mainly enriched in focal adhesion and Ca signaling pathways, which played pivotal roles in controlling skeletal muscle contraction and bone Ca transport. According to the GO and KEGG pathway analyses of DEGs, a total of 14 DEGs (Table 5) related to bone health were selected and verified by qRT-PCR.

Validation of DEGs by qRT-PCR

To validate the RNA-seq results, 14 DEGs containing 7 upregulated genes (COL6A2, TNN, PDGFA, COL6A3, COL6A1, ADRA1D, and BDKRB1) and 7 downregulated genes (MYLK2, EGF, CAV3, TNNC2, TNNC1, CACNA1S, and RYR3) were quantified using qRT-PCR. The mRNA expression level results of DEGs were normalized with reference gene 18S, and RNA-seq results were consistent (Figure 7A). The mRNA expression levels of these DEGs in NK and FK bones showed consistency with RNA-seq results (Figure 7B).

DISCUSSION

KBFs are severe welfare and health problem in laying hens. KBFs cause physiological stress, leading to elevated blood and feather CORT levels in laying hens (Rokavec and Šemrov, 2020; Wei et al., 2022). Similarly, the concentration of serum CORT in FK laying hens was higher than NK laying hens in this study, which demonstrated that KBFs induced stress.

Ca and P are essential nutrients regulating the bone quality and eggshell formation in poultry. Blood Ca and P levels can reflect the metabolic balance of Ca and P in the body, and their concentration in a normal range is important to maintain the calcification and remodeling of bone (Proszkowiec-Weglarz and Angel, 2013). Previous studies found that dietary Ca deficiency induced bone metabolism disorder and altered blood Ca and P levels in chickens (Jiang et al., 2013; Dijkslag et al., 2021), reducing bone quality and causing bone damage (Xu et al., 2020). Furthermore, 25-OHD$_3$ and 1,25-(OH)$_2$D$_3$, two important metabolites of vitamin D$_3$, play significant roles in modulating Ca and P absorption and metabolism in the intestinal tract and improving bone health and growth performance of animals (Kakhki et al., 2019). In laying hens and broilers, the supplementation of dietary 25-OHD$_3$ or 1,25-(OH)$_2$D$_3$ as a nutritional component can enhance bone strength and eggshell quality (Kakhki et al., 2019; Wu et al., 2022). This study showed that FK birds decreased Ca, P, and 25-OHD$_3$ concentrations in serum samples compared to NK layers, suggesting that reduced Ca, P, and 25-OHD$_3$ levels in the blood circulation might be associated with decreased keel bone mass and quality by inhibiting the mineralization of keel bone and increasing the fragility of keel bone in laying hens. PTH and CT play key regulatory roles in bone development and metabolism (Naot and Cornish, 2008; Wang et al., 2020). In general,

Table 4. Reads quality statistics after filtering sequencing data in normal keel (NK) and fractured keel (FK) bones.

Sample	Raw reads	Clean reads	Clean reads (%)	Q30 (%)	Mapped reads	Mapped reads (%)
NK1	41,775,692	38,912,544	93.14	92.95	35,943,105	92.37
NK2	36,352,878	33,951,292	93.39	92.38	31,277,562	92.12
NK3	39,340,382	36,964,610	93.96	91.78	33,757,817	91.32
NK4	39,683,608	36,959,450	93.13	91.26	33,879,654	91.67
FK1	40,909,132	38,341,178	93.72	92.61	35,224,457	91.87
FK2	36,080,296	33,658,470	93.28	92.28	31,125,509	92.47
FK3	42,837,796	39,971,388	93.30	92.55	36,868,701	92.24
FK4	40,711,558	38,127,104	93.65	92.90	35,303,765	92.59

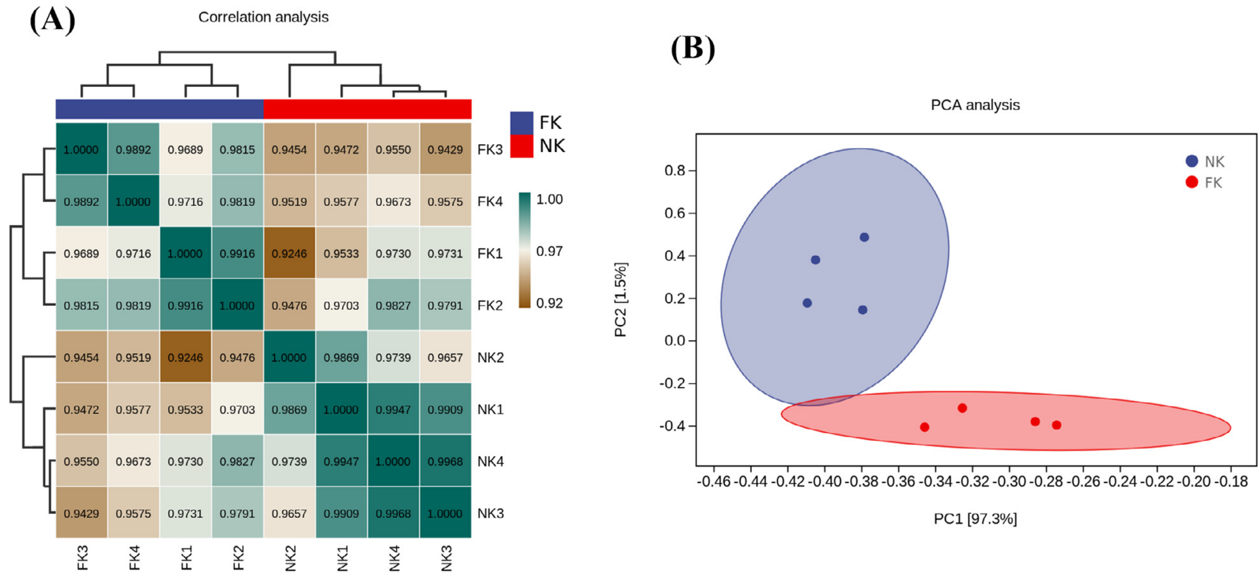

Figure 4. Results of (A) correlation analysis and (B) principal component analysis (PCA) of normal keel (NK) and fractured keel (FK) bone samples by RNA-seq analysis in laying hens.

hypocalcaemia during the eggshell mineralization caused by insufficient Ca stimulates the synthesis of PTH to increase the concentration of 1,25-$(OH)_2D_3$ and Ca and promote bone absorption (Singh et al., 1986). In this study, FK laying hens had high serum PTH levels, but 1,25-$(OH)_2D_3$ was similar in NK and FK hens, indicating that the lack of Ca could stimulate PTH in FK birds, which alleviates the loss of bone mass caused by Ca deficiency and maintains normal bone metabolism. CT can inhibit osteoclast activity and bone resorption by reducing the level of blood Ca (Naot and Cornish, 2008). In the present study, the concentration of serum CT and Ca in FK laying hens was lower than in NK laying hens, which suggested that reduced CT in FK birds might mitigate the adverse effect of low Ca level on bone remodeling. Because insufficient Ca content reduced bone mineralization and even lead to osteoporosis (Proszkowiec-Weglarz and Angel, 2013).

The balance between bone formation regulated by osteoblasts and bone resorption maintained by osteoclasts during the bone remodeling process plays a vital function in normal bone metabolism. ALP and OC are two typical indicators reflecting osteoblast activity, and their levels are related to osteoblast-regulated bone formation (Seibel, 2006). Previous studies showed that the levels of serum ALP and OC were significantly increased after bone injury, reflecting increased osteoblast activity (Hatayama et al., 2012; Kubo et al., 2012). TRAP is a common index used to evaluate the activity of osteoclasts and macrophages during bone remodeling. Angel et al. (2000) reported that reduced TRAP activity could inhibit the ossification of cartilage tissue, while increased

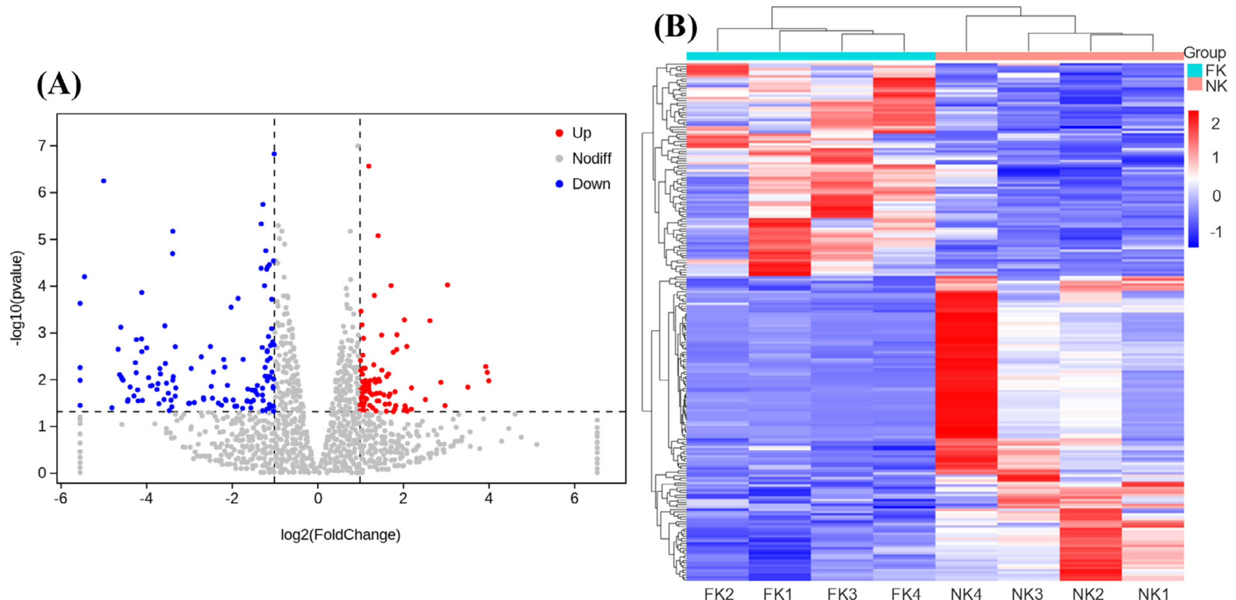

Figure 5. Results of (A) volcano plot and (B) clustering map of differentially expressed genes (DEGs) between normal keel (NK) and fractured keel (FK) bones by RNA-seq analysis in laying hens.

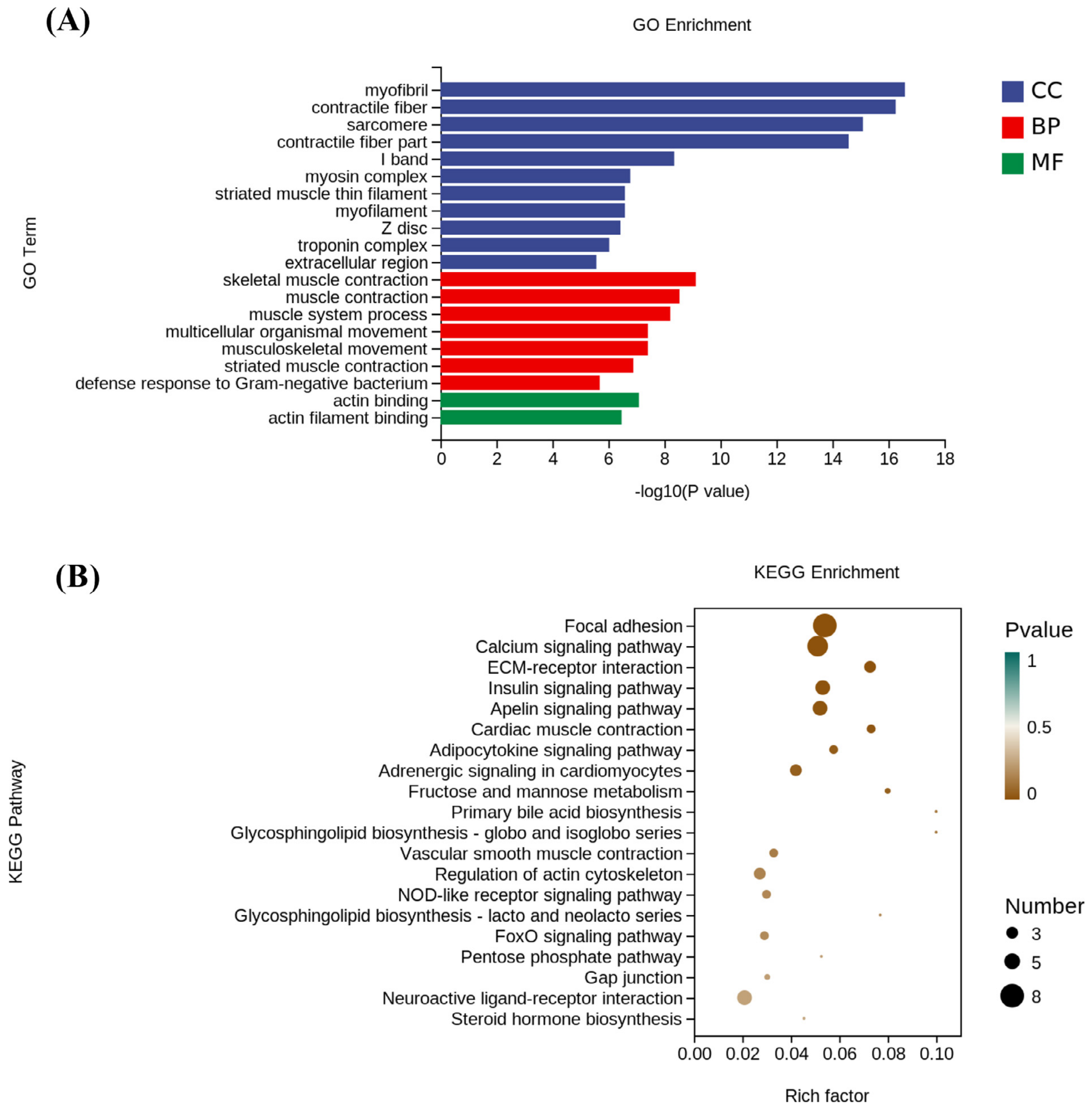

Figure 6. Results of (A) GO and (B) KEGG enrichment analysis of differentially expressed genes (DEGs) between normal keel (NK) and fractured keel (FK) bones by RNA-seq analysis in laying hens.

levels accelerated bone metabolism, leading to osteoporosis. This study found that the activities of ALP and TRAP and concentration of OC in serum samples of FK laying hens were reduced compared to NK laying hens, indicating that keel bone turnover of FK birds was lower than NK birds. TRAP staining displayed an increased number of osteoclasts in fractured keel bones, which suggested that abnormal bone metabolism was associated with keel bone fractures in laying hens. OPG is also a regulator of bone metabolism. In bone, OPG is mainly produced by osteoblast cell line cells and has a strong inhibitory effect on osteoclast formation (Bae and Kim, 2010). Blood OPG levels can reflect bone OPG activity, and people with the bone disease have an elevated level of blood OPG (Martini et al., 2007). In this study, the concentration of serum OPG in FK hens was significantly increased compared to NK hens, which could indicate that keel OPG level in FK birds was higher than that in NK birds. Elevated OPG levels in FK bone might be to inhibit osteoclast activity and reduce bone absorption, promoting bone formation and maintaining normal bone remodeling.

Micro-CT is applied to assess the bone health state of birds and other small animals, and it can provide various parameters related to bone strength and mass, such as cortical and trabecular bone parameters, and BMD (Bouxsein et al., 2010). BMD can detect the gain and loss changes of bone mass and can reflect bone mineralization, thereby it is a reliable predictor of bone health status in animals and humans (Barreiro et al., 2009). Some studies found the individuals with improved bone quality had elevated BMD (Wen et al., 2019), but bone with diseases or low quality had a reduced BMD (Teng et al., 2020). In laying hens, our previous study found

Table 5. Differentially expressed genes (DEGs) between normal and fractured keel bones enriched in KEGG pathway.

KEGG pathway	Number of DEGs	Pathway *P*-value	Gene ID	Gene name (abbreviation)	DEGs log2 (foldchange)	DEGs *P*-value
Focal adhesion	8	0.00099	ENSGALG00000039216	Collagen type VI alpha 2 chain (COL6A2)	1.221	0.011
			ENSGALG00000004538	Tenascin N (TNN)	1.156	0.048
			ENSGALG00000003642	Platelet derived growth factor subunit A (PDGFA)	1.090	0.015
			ENSGALG00000003923	Collagen type VI alpha 3 chain (COL6A3)	1.081	0.038
			ENSGALG00000005974	Collagen type VI alpha 1 chain (COL6A1)	1.045	0.021
			ENSGALG00000006273	Myosin light chain kinase 2 (MYLK2)	−4.432	0.030
			ENSGALG00000012155	Epidermal growth factor (EGF)	−2.946	0.006
			ENSGALG00000008351	Caveolin 3 (CAV3)	−1.881	0.038
Calcium signaling pathway	7	0.00292	ENSGALG00000038787	Adrenoceptor alpha 1D (ADRA1D)	1.836	0.037
			ENSGALG00000020386	Bradykinin receptor B1 (BDKRB1)	1.240	0.011
			ENSGALG00000006273	Myosin light chain kinase 2 (MYLK2)	−4.432	0.030
			ENSGALG00000006835	Troponin C2 (TNNC2)	−4.196	0.017
			ENSGALG00000001459	Troponin C1 (TNNC1)	−2.984	0.033
			ENSGALG00000037744	Calcium voltage-gated channel subunit alpha1 S (CACNA1S)	−1.550	0.041
			ENSGALG00000009705	Ryanodine receptor 3 (RYR3)	−1.276	0.016

that dietary soybean oil supplementation-induced bone fractures were related to reduced BMD (Wei et al., 2021b). Study also reported that BMD elevated during fracture healing process (Gao et al., 2013). In this study, FK bones reduced BMD in laying hens, which indicated that keel bone fractures were likely related to BMD variations.

In the present study, RNA-seq was performed to identify potential DEGs in NK and FK bones, which regulated bone health and metabolism in laying hens. Fourteen DEGs between NK and FK bones enriched in focal adhesion and Ca signaling pathway were identified by KEGG pathway analysis (Table 5). These analyses identified DEGs, including 7 upregulated genes (COL6A2, TNN, PDGFA, COL6A3, COL6A1, ADRA1D, and BDKRB1) and 7 downregulated genes (MYLK2, EGF, CAV3, TNNC2, TNNC1, CACNA1S, and RYR3), that play essential roles in keel bone health and quality, were verified by qRT-PCR. The results of qRT-PCR verification were consistent with the RNA-seq results, demonstrating the reliability of RNA-seq results.

In the identified 14 DEGs, COL6A1, COL6A2, COL6A3, TNN, PDGFA, EGF, MYLK2, and CAV3 were enriched in the focal adhesion pathway. Type VI collagen is non-fibrous collagen that is the major structural component of microfibrils in most tissues (Lampe and Bushby, 2005) and is associated with the collagen-binding domain of cartilage matrix proteins (Koller et al., 1989). COL6A1, COL6A2, and COL6A3 are three basic structural subunits of type VI collagen, which play a key regulatory role in muscle tissue development (skeletal and pectoral muscles) (Listrat et al., 1999), and these genes dysfunction induces muscle diseases (Braghetta et al., 2008). CAV3 is an animal muscle-specific protein marker stably expressed in cardiac, skeletal, and smooth muscles, and its expression disorder could impair muscle function (Shang et al., 2017). At the transcriptional level, the expression levels of COL6A1, COL6A2, and COL6A3 were upregulated, and CAV3 was downregulated in FK bones, which suggested that abnormal expression of these genes related to muscle function reflected the association between muscle function and

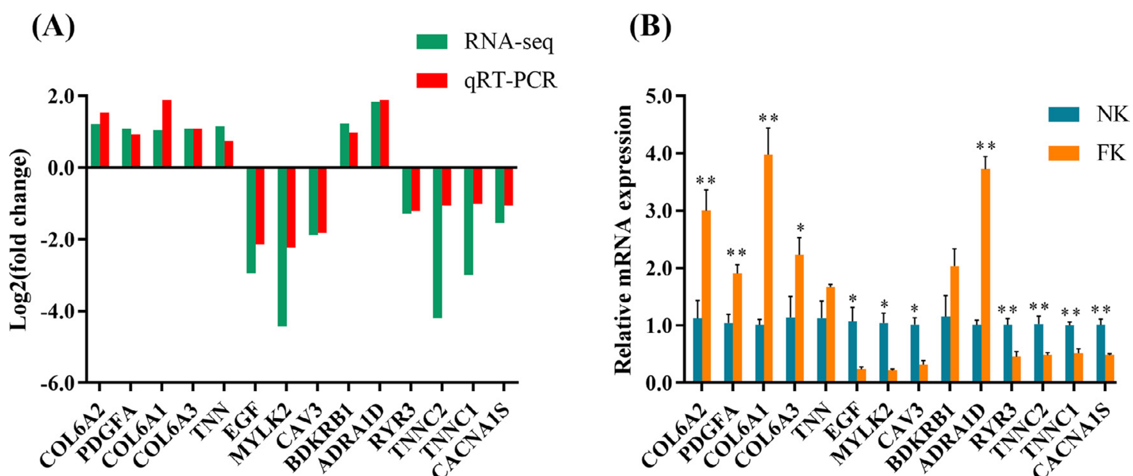

Figure 7. qRT-PCR validated the expression patterns of candidate genes involved in bone health in laying hens. (A) Comparison of expression levels of candidate genes as per RNA-seq and RT-qPCR analysis. (B) Relative mRNA expression of 14 screened genes in normal keel (NK) and fractured keel (FK) bones was detected by qRT-PCR.

keel bone health. KBFs impair the mobility of laying hens (Rentsch et al., 2019), thereby reducing muscle contraction and triggering abnormal expression of associated genes, which might also be conducive to fracture healing. TNN is a glycoprotein secreted into the extracellular matrix of developing bones, and it can effectively promote the formation of new bone and has a positive regulatory effect on osteoblasts (Meloty-Kapella et al., 2008). In chickens, TNN is mainly located in the developing skeleton, and its expression is relatively conserved, implying that it plays a crucial role in osteogenesis (Meloty-Kapella et al., 2008). The expression of TNN in FK bones was significantly higher than in NK bones in this study, which might suggest that upregulated TNN in FK bones could be to improve the activity of osteoblasts in fractured keels, accelerating bone formation and promoting the repair of damaged bone in laying hens. EGF is an important intestinal epithelial growth repair factor that regulates cell metabolism, growth, and differentiation and repairs damaged intestinal mucosa (Hodges et al., 2012). In this study, EGF expression was significantly downregulated in FK bones, which implied that keel bone fractures might impair intestinal mucosa of laying hens.

The remaining seven DEGs, including ADRA1D, BDKRB1, TNNC2, TNNC1, CACNA1S, MYLK2, and RYR3, were enriched in the Ca signaling pathway. Ca signaling plays an essential role in the development, regulation, and regeneration of bone and skeletal muscle because of the key role of Ca^{2+} (Tu et al., 2016). Troponin in the Ca signaling pathway regulates bone development, which is composed of three subunits, namely troponin C (**TNNC**, Ca^{2+}-binding subunit), troponin I (**TNNI**, inhibitory subunit), and troponin T (**TNNT**, tropomyosin-binding subunit). A study has reported that TNNC (e.g., TNNC2) plays an important biological function in the regulation of healthy development and regeneration of the skeletal system, and the expression of TNNC at the transcript level in individuals with ankylosing spondylitis was significantly lower than normal individuals (Yu et al., 2020). This result was similar to our finding that the expression of TNNC (e.g., TNNC1 and TNNC2) in FK bones was reduced compared to NK bones, indicating that TNNC also modulated keel bone health in laying hens. Furthermore, ADRA1D and MYLK2 can maintain smooth muscle and skeletal muscle contraction (Lee et al., 1992; Docherty, 2010). This study showed that FK bones upregulated ADRA1D and downregulated MYLK2 expression, which might imply that the disorder of skeletal muscle contraction was likely related to KBFs in laying hens. RYR is an intracellular protein found in intracellular calcium release channels and is present in muscle and non-muscle tissues (Fill and Copello, 2002). Since Ca^{2+} is released from the sarcoplasmic reticulum or endoplasmic reticulum, the intracellular Ca^{2+} storage is mediated by RYR and inositol triphosphate receptors. RYR has three subtypes include RYR1, RYR2, and RYR3. RYR1 and RYR2 are subtypes of ryanodine receptors mainly located in skeletal muscle and myocardium and are involved in regulating Ca^{2+} conduction. RYR3 is distributed in various tissues and is co-expressed with RYR1 and RYR2. Besides, RYR3 is similar to RYR2 in that it can be activated by Ca^{2+} (Ogawa et al., 2000). RYR3 may participate in the transmission of Ca^{2+} signaling in smooth muscle and other non-muscle cells through the Ca^{2+}-induced Ca^{2+} release pathway. In this study, the expression of the RYR3 gene at the transcriptional level in FK bones was significantly lower than that in NK bones, which indicated that bone fractures could affect Ca^{2+} signaling in keels and lead to the imbalance of intracellular ion homeostasis.

On the other hand, bone repair after fractures is a special process in which a series of cellular and molecular events occur to generate new bone, and various proteins and growth factors regulate the formation, ossification and function of new bone in this process (Arvidson et al., 2011). Previous studies reported that the expression of bone collagen (collagen type III, VI, etc.), tenascin N, troponin, growth factors released from platelets, osteonectin, and EGF promoted bone matrix synthesis, bone formation and biomineralization during fracture healing (Meloty-Kapella et al., 2008; Arvidson et al., 2011; Yu et al., 2020). In this study, the expression of COL6A1, COL6A2, COL6A3, PDGFA, and TNN was upregulated and that of TNNC1, TNNC2, and EGF was downregulated in FK bones in comparison to NK bones, which might indicate that these DEGs enriched in focal adhesion and Ca signaling pathway identified by RNA-seq were also involved in regulating keel fracture healing in laying hens.

CONCLUSION

This study showed that the levels of serum Ca/P metabolism- and bone remodeling-related indexes in laying hens with keel bone fractures were significantly altered, and fractured keel bones reduced bone mineral density. Besides, RNA-seq revealed that the DEGs associated with muscle contraction and Ca^{2+} transport enriched in focal adhesion and Ca signaling pathway could modulate keel bone health and quality. Therefore, the results of this study suggest that the changes in bone structure and metabolism are likely related to keel bone fractures and fracture healing in laying hens.

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DISCLOSURES

The authors confirm that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102438.

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