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Ovary Transcriptome Profiling in Broody and Egg-laying Chahua Chickens

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Broodiness in egg-laying hens (EHs) leads to ovarian atrophy, resulting in reduced egg-laying performance. However, the ovarian regulatory mechanisms in broody hens (BCs) remain elusive. Therefore, ovaries were removed from 300-day-old BCs and EHs for RNA sequencing. Ovarian morphology and histological characteristics of the BC and EH groups were compared and analyzed. The EH group had significantly more hierarchical follicles (HFs) and small yellow follicles (SYFs) than that of the BC group. Although several secondary follicles (SFs) and primary follicles were observed in the ovaries of the EH group, only a few SFs were observed in the ovaries of the BC group. Subsequently, RNA-sequencing analysis was conducted to determine the ovarian expression profiles of the two groups. Transcriptome sequencing identified 259 differentially expressed genes (DEGs) between the BC and EH groups. Of the 259 DEGs, 136 were upregulated and 123 were downregulated. The DEGs were mapped to 22 gene ontology terms and 4 Kyoto Encyclopedia of Genes and Genomes pathways for ovarian tissue. The analysis showed that matrix metalloproteinases 11/13 (MMP11/MMP13) were enriched in the extracellular matrix. The extracellular matrix mediated by MMP13 is affected by follicle-stimulating hormone, prolactin, and estrogen, which are critical signaling pathways that may affect ovarian follicle development to regulate the large yellow follicle reserve process and the ovulation cycle of broody Chahua chickens. These findings indicate that understanding differences in gene expression between the ovarian tissues of BCs and EHs could serve as a valuable reference point for enhancing egg-laying performance in Chahua chickens.

Key words: broody hens, ovary, transcriptome analysis, reverse transcription-qPCR, western blot

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Introduction

Chahua chickens, a popular breed in China, are a rare tropical primitive species that have been domesticated and bred from the red *Gallus gallus*. These birds are typically found in the tropical or subtropical regions along the southern borders of China. This species exhibits early sexual development, robust feeding tolerance, strong disease resistance, and tender flesh[1].

Brooding is a maternal behavior in hens that is characterized by a reduced intake of food and water, frequent access to the nest, increased egg hatching, and cessation of ovulation[2,3]; these changes have a significant impact on egg-laying performance. The development of the ovary involves recruitment, selection, dominance establishment, growth promotion, and maturation prior to ovulation[4]. Based on their diameter, follicles are generally divided into white follicles, small yellow follicles (SYFs), and hierarchical follicles (HFs) with diameters of < 4, 4–8, and > 8 mm, respectively[5]. To maintain continuous ovulation, a SYF is selected and enters the HF layer daily, which then begins to grow rapidly and eventually differentiates[6]. The follicle reserve at each stage may affect the ovulation cycle and, ultimately, the ovulation rate[7]. Broodiness results in the cessation of ovulation and ovary development, which is associated with the absence of SYFs and HFs[8]. The ovarian function of follicular development and normal ovulation is maintained by follicle-stimulating hormone (FSH), prolactin (PRL), and estradiol (E_2)[9].

In recent years, RNA sequencing has attracted significant attention and has been used to study gene expression and regulation, and to explore new breeding genes[10,11]. Most research has focused on the use of high-throughput sequencing to study the mechanisms of high and low egg production in the hypothalamus, pituitary gland, and ovaries[12–14]. However, there are no

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reports on the involvement of mRNAs in the ovaries of broody hens.

In this study, ovarian tissues from 300-day-old egg-laying hens (EHs) and broody chickens (BCs) were subjected to transcriptome analysis, which generated mRNA profiles of the ovaries. Next, a comparative analysis of the mRNA transcriptomes was performed. The resulting data was used to further understand BC ovarian tissues and to study mRNA regulatory mechanisms in poultry.

Materials and Methods

Animal experimentation ethical statement

All animal experimental procedures were approved and guided by the Kunming University Animal Care and Use Committee (approval ID: kmu2023012; publication date: January 1, 2024).

Experimental animal, sample collection, and preparation

Chahua chickens used in this study were obtained from Xishuangbanna Chahua Chicken Industry Development Co., Ltd. (Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China). During the experimental period, the nutritional level and feeding conditions were maintained in accordance with standard commercial hen food and the animals had free access to water. A total of 1848 hens from the same generation were selected and individually housed in cages. At 300 d of age, birds with similar appearances and genetic backgrounds were randomly assigned to two groups: the broody group (BC) and the egg-laying (EH) group. EHs exhibited typical patterns, including daily egg-laying time and ovipository cycles. In contrast, broody birds engaged in persistent nesting and incubation for approximately 25–30 consecutive days, resulting in atrophic ovaries.

Ovarian histological features and plasma hormone analyses

Twenty-four hens were euthanized at the age of 300 d; 12 hens were randomly selected from each group (BC or EH). Ovarian tissues were divided into two parts. One part was rapidly frozen in liquid nitrogen for subsequent RNA extraction, and the other part was fixed in 10% paraformaldehyde for 24 h, dehydrated, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. The sections were then observed under a microscope. Additionally, 2 mL of venous blood was collected from each of the 48 hens (24 from each group), and subjected to centrifugation at 13,400 × *g* for 15 min. The plasma was extracted and stored at –20 °C. Plasma concentrations of PRL, E_2, and FSH were measured using chicken-specific ELISA kits (Yuanye Bio-Technology, Shanghai, CN), following the manufacturer's instructions.

Transcriptome sequencing

Total RNA was extracted from six ovarian tissue samples (three randomly selected from each group (BC or EH)) using an RNeasy Mini Kit (Takara, Beijing, CN). The degree of RNA degradation was assessed using agarose gel electrophoresis. The purity of RNA was analyzed using the NanoPhotometer® (IMPLEN, CA, USA), while RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Subsequently, cDNA libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA), and the quality of the libraries was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technology, CA, USA).

Quality control, alignment, and annotation

The imaging data obtained from Illumina's CASAVA (version 1.8) were subjected to high-throughput quenching and converted into FASTQ-format sequence data (reads). Initially, in-house Perl scripts were used to process raw FASTQ-format data. The filtering process involved the removal of reads containing adapters, low-quality reads, and reads with indeterminate base data. Concurrently, the Q20, Q30, and GC content of the clean data were assessed. The genome sequence of the chicken was obtained from the genome website (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000002315.4_Gallus_gallus-5.0_genomic.gff.gz), and a reference genome index was constructed using Hisat2 (version 2.0.5). Clean reads were then mapped to the reference genome.

Bioinformatics analysis

Normalized fragments per kilobase of exon model per million mapped reads (FPKM) were used to estimate gene expression levels. Hierarchical clustering was performed using the PheatMap package (version 1.0.12) in R, while principal component analysis of FPKM values was conducted using Origin software (version 2019b). Pearson's correlation coefficient was calculated using Origin software to determine the correlation of FPKM values among samples. Differential expression analysis of both combinations was performed based on raw read counts using the DESeq2 R package (version 1.16.1). The Benjamini-Hochberg approach was applied to adjust *P* values. Differential expression was considered significant at an adjusted *P* value < 0.05 and |log2foldchange| > 2. Gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the differentially expressed genes (DEGs) was performed using ClusterProfiler. Statistical significance was defined as a *P*-value < 0.05.

Verification by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), immunofluorescence (IF), and western blotting (WB)

Ten potential samples were randomly selected for RT-qPCR to validate the accuracy of the transcriptome sequencing data. Total RNA was extracted from ovarian tissues (EH and BC groups) using an RNeasy Mini Kit (Takara, Beijing, CN). The NanoPhotometer® (IMPLEN, CA, USA) was used to assess the total RNA content and quality, while 1% agarose gel electrophoresis was employed to evaluate the overall RNA quality. For RT-qPCR, the PrimeScript™ RT reagent Kit was used with gDNA Eraser (Takara, Beijing, CN), following the manufacturer's protocols. In Step 1, a reaction mixture (10 μL) containing 2 μL gDNA Eraser, 2 μL 5× gDNA Eraser Buffer, RNase Free dH_2O, and 1 μg total RNA was prepared to remove genomic DNA. The mixture was subjected to a PCR procedure at 42 °C for 2 min. In Step 2, a reaction mixture (20 μL) consisting of 10 μL of the reaction liquid from Step 1, 1 μL RT Primer Mix, 1 μL PrimeScript RT Enzyme Mix I, 4 μL RNase Free dH_2O, and 4 μL 5× PrimeScript Buffer

2 was used for cDNA synthesis. The PCR procedure included incubation at 37 °C for 15 min, followed by a short incubation at 85 °C for 5 s. In Step 3, a reaction mixture (20 μL) containing 10 μL SYBR@ Premix Ex TaqII (2×), specific primers (0.8 μL, 10 μM), 0.4 μL ROX Reference II, 6 μL dH_2O, and 2 μL cDNA was prepared for RT-qPCR. The PCR procedure included an initial activation step at 94 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. Actin beta was used as the endogenous reference gene for normalization, and the $2^{-\Delta\Delta CT}$ method was used to determine gene expression levels. The sequences of primers are listed in Supplementary Table S1.

To identify candidate genes related to egg-laying performance, DEGs related to reproduction, including matrix metalloproteinase 13 (MMP13), were selected for IF and WB. Ovarian tissues collected above were fixed in 10% paraformaldehyde for 24 h, dehydrated, embedded in paraffin, sectioned, dewaxed in xylene, antigen repaired, and blocked with serum. Mouse monoclonal antibodies against MMP13 (Thermo, New York, USA, 1:1000) were added and incubated overnight, followed by the addition of Cy3-labeled fluorescent secondary antibody (Thermo, New York, USA, 1:1000) for 1 h. 4′,6-diamidino-2-phenylindole was added to stain nuclei and images were collected using a fluorescence microscope. The large intestinal tissue of Chahua chickens was used as a negative control. Total protein was extracted from randomly selected ovarian tissues of three BC and three EH Chahua chickens, using a Rapid Cell Lysis Kit (Solarbio, Beijing, CN). A bicinchoninic acid protein quantification kit (Tiangen, Beijing, CN) was used to determine protein concentrations. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing and denaturing conditions. Subsequently, proteins were transferred to a polyvinylidene fluoride membrane (PVDF). The membrane was incubated with a 5% bovine serum albumin/Tris-buffered saline with 0.1% Tween® 20 (TBST) solution and agitated for 1 h. A mouse monoclonal antibody against MMP13 (Thermo, New York, USA, 1:1000) was separately added to the membrane and agitated for 30 min, followed by overnight incubation at 4 °C. The membrane was washed three times with TBST (10 min per wash). Goat anti-mouse immunoglobulin G antibody (Thermo, New York, USA, 1:1000) was added and the membrane was incubated on a shaker for 1 h. Three additional washes with TBST were performed for 10 min each. Finally, the PVDF membrane was subjected to chemiluminescence exposure to obtain the experimental results.

Data statistics and analysis

For follicle counts, plasma hormone concentrations, and WB analysis, differences between groups were evaluated using the *t*-test ($P < 0.05$). All data are presented as the mean ± the standard error of the mean.

Results

Comparison of histological characteristics and plasma hormones

The density of cells in the ovary cortex and medulla of BCs was greater than that of EHs (Figure 1A, 1B). HFs were not observed in BCs, but significantly more SYFs were observed in the EH group (Table 1, $P < 0.01$). Ovarian tissue sections of the two groups were microscopically observed under the same magnifi-

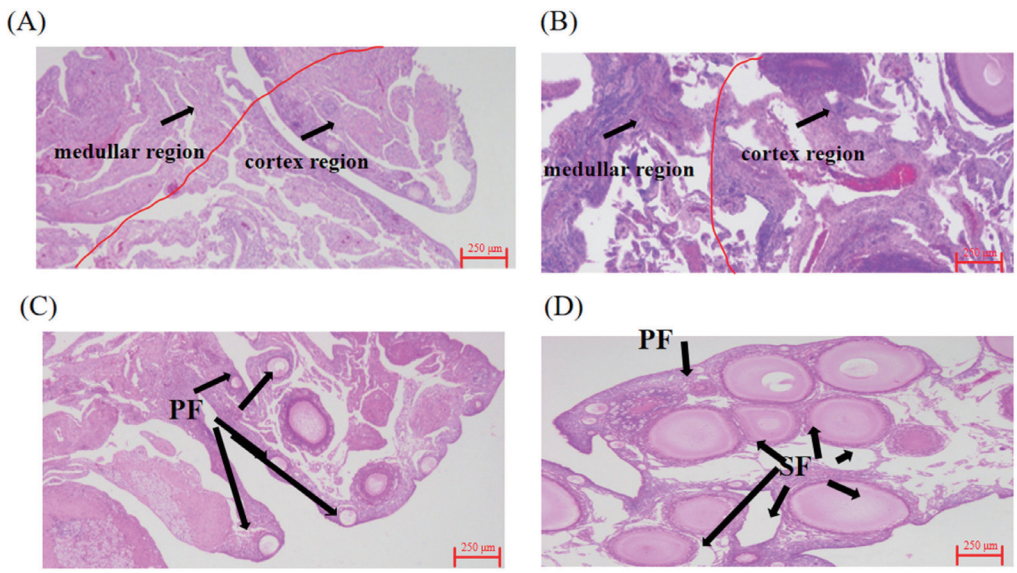

Fig. 1. **Histological characteristics in ovaries of (A,C) BCs and (B,D) EHs (HE staining at 40×).** Secondary follicles (SFs, > two layers of granulosa cells) and primary follicles (PF, one layer of cuboidal granulosa cells). The red line is an approximate junction, the right is the cortex region, and the left is the medullar region. BC, broody chickens; EH, egg-laying hens; HE, hematoxylin and eosin.

Table 1. **Comparison of ovarian morphological characteristics between egg-laying and broody chickens.**

	HFs (> 8 mm, count)		SYFs (4~8 mm, count)		WFs (1~4 mm, count)	
Chickens	BC	EH	BC	EH	BC	EH
Mean value	\	4.33 ± 1.01	1.03 ± 0.09	7.13 ± 0.42	20.26 ± 2.14	23.33 ± 3.68
P value			2.12E-11**		0.32	

Note: *$P < 0.05$, **$P < 0.01$; HFs (hierarchical follicles, > 8 mm in diameter), SYFs (small yellow follicles, 4~8 mm in diameter), and WFs (white follicles, 1~4 mm in diameter).HF was not observed in the BC group. EH, egg-laying hens; BC, broody chickens.

Table 2. **Plasma concentrations of follicle stimulating hormone (FSH), estradiol (E_2) and prolactin (PRL) between EHs and BCs.**

	PRL (mIU/mL)		E_2 (pg/mL)		FSH (mIU/mL)	
Chickens	BCs	EHs	BC	EH	BC	EH
Mean value	21.05 ± 0.06	16.53 ± 0.05	69.04 ± 4.2	74.24 ± 3.6	6.04 ± 0.7	9.07 ± 0.32
P value	0.003**		0.0066**		0.04*	

Note: *$P < 0.05$, **$P<0.01$; BCs, broody chickens; EHs, egg-laying hens.

Table 3. **RNA-sequencing reads and mapping rate of ovary tissues from EHs and BCs.**

Sample	Raw_reads	Clean_reads	Clean_bases	Q20 (%)	Q30 (%)	GC (%)	Total_map (%)
BC1	46108296	44144790	6.62G	98.73	96.16	50.62	91.43
BC2	52525826	50890726	7.63G	97.22	93.18	53.37	90.12
BC3	52330196	50689546	7.6G	98.67	96.01	49.32	90.78
EH1	46408830	44754640	6.71G	97.52	93.53	54.17	93.12
EH2	47920646	46598038	6.99G	97.47	93.37	52.19	92.01
EH3	48038886	46654536	7.0G	97.46	93.11	49.95	92.66

Note: BCs, broody chickens; EHs, egg-laying hens.

cation, which revealed only a few secondary follicles (SFs) in the ovaries of BCs (Figure 1C), but many SFs and primary follicles in the ovaries of EHs (Figure 1D). The plasma concentrations of PRL were significantly higher in the BC group than those in the EH group ($P < 0.01$), whereas FSH and E_2 levels were significantly higher in the EH group than those in the BC group (Table 2, $P < 0.05$).

Data summary of the ovary transcriptome in Chahua hens

Six ovarian cDNA libraries, including EH and BC, were constructed from Chahua chickens. Each library contained more than 46 million raw and 44 million clean reads. The clean reads had quality levels of 97.22% in Q20 and 93.11% at Q30, respectively. With the average GC content of the clean reads at 50.95%, 90.12%–93.12% of sequences were mapped to the genome (Table 3). FASTQ files were submitted to the Sequence Read Archive database under the BioProject ID PRJNA979785.

Analysis of DEGs and functional enrichment

There were 259 DEGs, including 136 that were upregulated and 123 that were downregulated in the ovaries of BC and EH Chahua hens (Figure 2A, Supplementary Table S2). Samples from the same group clustered together and the heat map visually reflected gene expression patterns of the BC and EH groups (Figure 2B).

GO and KEGG pathway enrichment analyses were performed to elucidate the biological functions of the DEGs. A total of 22 significantly enriched GO terms ($P < 0.05$) were obtained, where approximately 7 significant terms were found to be enriched in biological processes mainly involved in multi-organism processes, positive regulation of response to stimulus, and regulation of phosphate metabolic processes. In the cellular component, three significant terms were enriched, with the main involvement in the extracellular region, extracellular region part, and extracellular matrix. In the molecular function process, 12 significant terms were enriched, with the main involvement in peptidase regulator activity and metalloendopeptidase activity, among others (Figure 3A, Supplementary Table S3). Genes corresponding to the extracellular region, extracellular region part, extracellular matrix, and metalloendopeptidase activity are shown in Table 4, where the extracellular matrix pathway (MMP11/MMP13) directly related to reproduction is enriched. Four significant KEGG pathways were obtained ($P < 0.05$), mainly involved in folate biosynthesis, the peroxisome proliferator–activated receptor (PPAR) signaling pathway, and cell adhesion molecules (CAMs) (Figure 3B, Supplementary Table S4). The genes corresponding to the above-mentioned KEGG pathways are listed in Table 4.

Verification of DEGs

To validate the RNA sequencing results, eight DEGs were randomly selected for RT-qPCR analysis. These included four upregulated genes (cytochrome P450, family 2, subfamily C, polypeptide 23a [*CYP2C23a*], fatty acid binding protein

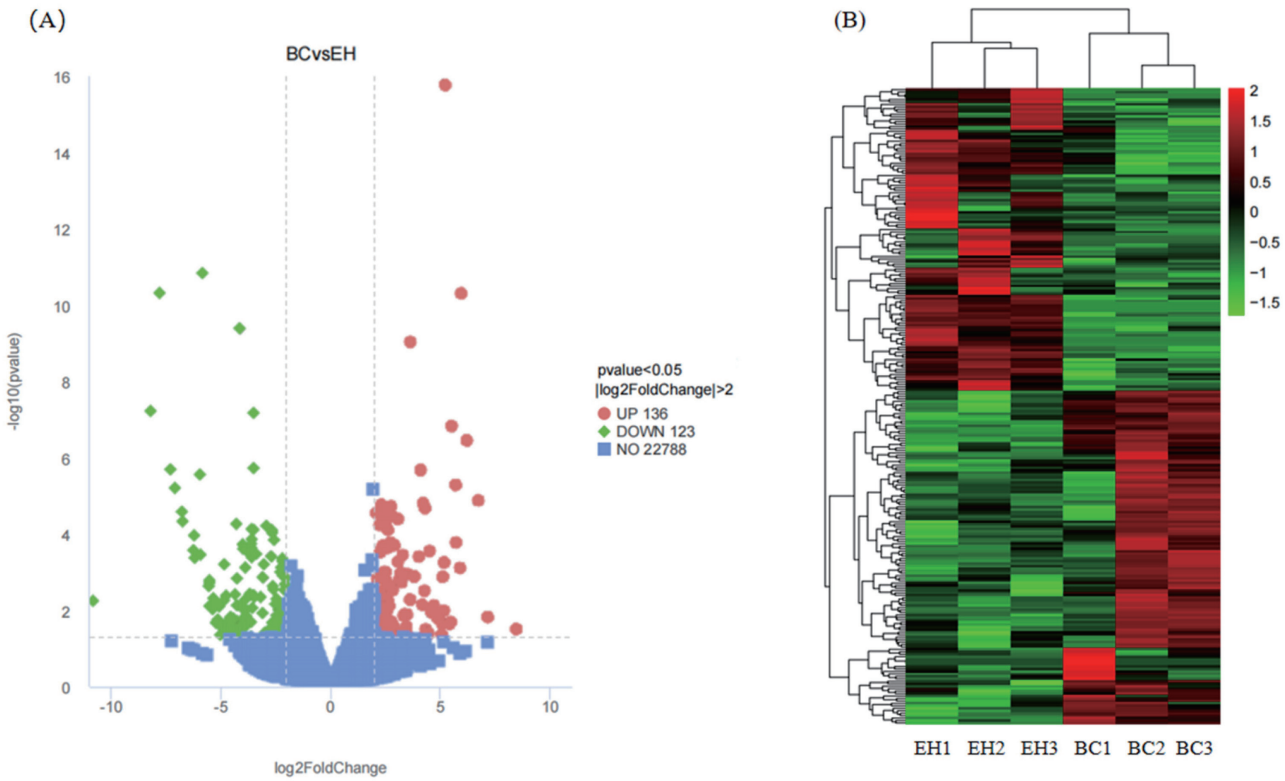

Fig. 2. **Differentially expressed genes (DEGs). (A) Volcano plot of DEGs; (B) Hierarchical cluster analysis of DEGs.** BC, broody chickens; EH, egg-laying hens.

1 [*FABP1*], FABP1, and apolipoprotein A4 [*APOA4*]) and four downregulated genes (*MMP13*, *MMP11*, relaxin 3 [*RLN3*], and tyrosine hydroxylase [*TH*]) in the BC group. The expression trends determined by RT-qPCR were consistent with the RNA sequencing results (Figure 4). IF showed that MMP13 was localized throughout the ovary (oocyte, yolk, granular cells, and theca cells) (Figure 5). IF and WB showed that MMP13 proteins were expressed in the ovarian tissues of Chahua chickens (Figure 5). MMP13 protein expression was higher in EHs than that in BCs, which was consistent with mRNA expression (Figure 5). IF staining of the negative control showed no positive signal or only a slight brown signal, which was lighter than that of the chicken ovarian tissue group (Figure 5).

Discussion

Brooding is a maternal behavior in poultry that reduces laying performance[15]. To provide a comprehensive view of the transcriptome-level changes that occur within the ovaries of BCs, transcriptome analysis was used to elucidate candidate gene functions and their regulatory effectors. In chickens, the hypothalamic-pituitary-gonadal axis mediates ovarian development via reproductive endocrine hormones, including gonadotropin-releasing hormone, PRL, FSH, E_2, and progesterone[9]. PRL is necessary for broodiness in poultry, as it acts as a negative regulator of reproductive activities by suppressing the secretion of gonadotropins and causing atresia of ovarian follicles[16]. PRL inhibits the release of gonadotropins and decreases FSH secretion[9]. Previous studies have shown that FSH stimulates the ovaries and causes ovulation[17]. In the current study, the levels of FSH and E_2 were significantly higher in the EH group than those in the BC group, and PRL was significantly higher in the BC group than that in the EH group. Therefore, FSH, E_2, and PRL levels may be immediate causes of broodiness in Chahua chickens.

Upon maturity and ovulation of F1 (the largest follicle) in HFs, F2 (the second-largest follicle) and F3 (the third-largest follicle) are considered the new F1 and F2 follicles. Additionally, SYFs are selected as the smallest F6 (the least mature follicle) [6], and HFs generally do not develop atresia[18]. Thus, reduced numbers of HFs and SYFs in the BC group might also be an important reason for broodiness.

Transcriptome analysis revealed 259 DEGs in the ovaries of the EH and BC groups, with 123 upregulated and 136 downregulated genes. KEGG pathway analysis showed that DEGs identified in the ovaries of the EH and BC groups were mainly involved in signaling pathways, such as folate biosynthesis, the PPAR signaling pathway, and CAMs. Folate improves symptoms and infertility in patients with polycystic ovary syndrome[19,20].

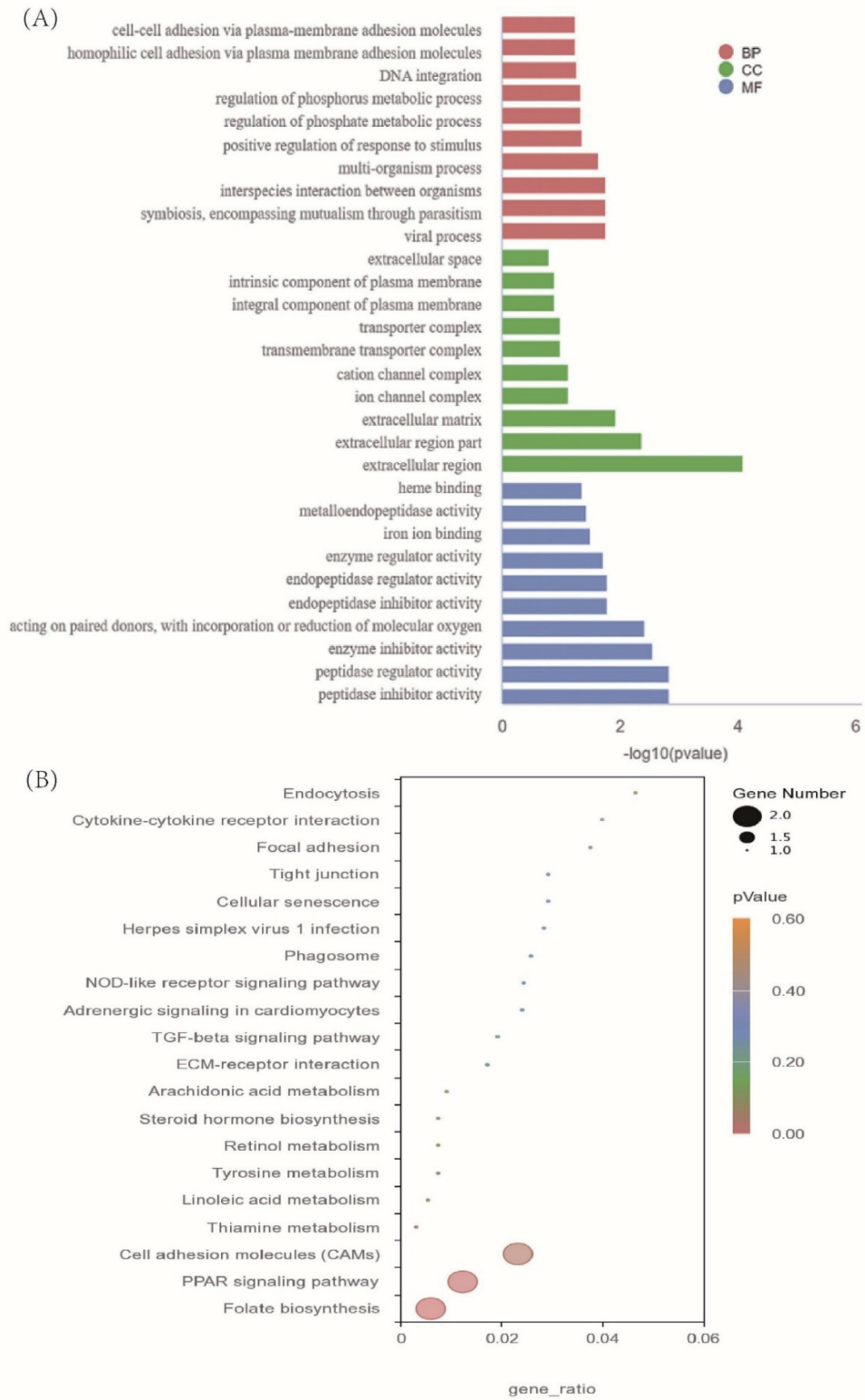

Fig. 3. **Differentially expressed genes (DEGs) functional enrichment in the ovaries of BCs and EHs.** (A) Gene ontology (GO) analysis of the top 30 DEGs. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the top 20 DEGs. BP, biological process; CC, cellular component; MF, molecular function; BCs, broody chickens; EHs, egg-laying hens; PPAR, peroxisome proliferator–activated receptor.

Table 4. **List of partially representative differentially expressed genes (DEGs).**

Title 1	Genes	Description	log2FC	FDR
extracellular region, extracellular region part, extracellular matrix	MMP13	matrix metallopeptidase 13	-3.61	7.48E-05
	MMP11	matrix metallopeptidase 11	-2.08	0.00080
	CARTPT	CART prepropeptide	-2.64	0.0023
	APOA4	apolipoprotein A4	3.21	0.0018
	RLN3	relaxin 3	-2.60	0.00013
metalloendopeptidase activity	MMP13	matrix metallopeptidase 13	-3.61	7.48E-05
	MMP11	matrix metallopeptidase 11	-2.08	0.00080
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	TH	tyrosine hydroxylase	-3.64	0.00024
	CYP2C23a	cytochrome P450, family 2, subfamily C, polypeptide 23a	2.03	0.015
peroxisome proliferator–activated receptor (PPAR) signaling pathway	FABP1	fatty acid binding protein 1	4.16	0.007
	FABP2	fatty acid binding protein 2	3.10	0.049
Cell adhesion molecules (CAMs)	MPZ	myelin protein zero	-3.79	0.00029
Folate biosynthesis	TH	tyrosine hydroxylase	-3.64	0.00024

The PPAR signaling pathway is involved in ovarian follicle development[21]. This pathway involves DEGs, such as FABP1 and FABP2. FABP1 affects ovarian development[22]. CAMs are involved in the cell transcriptome of Chinese hamster ovaries[23]. This pathway involved DEGs, such as myelin protein zero; however, there is no reported correlation with reproduction. Thus, these significantly enriched signaling pathways may affect egg laying and broodiness in chickens.

GO analysis showed that DEGs in the ovaries of the BC and EH groups were significantly enriched for metalloendopeptidase activity, extracellular matrix extracellular region, and extracellular region part. DEGs, such as *RLN3*, *MMP13*, and *MMP11*, were involved in the extracellular matrix, extracellular region, metalloendopeptidase activity, and extracellular region part. The relaxin gene is expressed in the reproductive tract, including the ovaries and uterus of mammalian species. RLN3 is involved in tissue remodeling during ovulation in birds[24]. RLN3 also plays an important role in follicular recruitment, which warrants further investigation[25]. Recent studies have also revealed a paracrine role of RLN3, which involves its binding to relaxin family peptide receptor 1 in ovarian theca cells, potentially initiating ovulation after a luteinizing hormone surge[26]. Therefore, *RLN3* may play an important role in regulating egg-laying performance. The extracellular matrix is closely related to animal reproduction, especially ovary development and ovulation[27]. Increased deposition of stromal collagen reduces ovulation ability, possibly due to increased collagen around the follicle, which forms a barrier to the proteolytic remodeling necessary for follicle formation. Follicle growth requires the remodeling of the surrounding extracellular matrix, leading to follicle rupture. During ovulation, induction of MMPs is required for collagen degradation and follicular rupture[28,29]. More than ovulation, follicle development, maturation, luteal formation, and degeneration in the reproductive cycle of the ovary are dependent on periodic degradation of the extracellular matrix and tissue reconstruction[30]. FSH, PRL, and ESR participate in the mechanisms that orchestrate extracellular matrix turnover during ovarian follicular development in hen ovaries by regulating the transcription, translation, and/or activity of some constituents of the MMP system[31–33]. MMP13 induction facilitates extracellular matrix remodeling during ovarian cycling[32]. MMP13 also influences ovarian follicular development[32]. Therefore, FSH, PRL, and ESR may participate in the mechanisms orchestrating extracellular matrix turnover mediated by MMP13 in the ovaries of Chahua hens, regulating ovarian development and ovulation, which affects broodiness (Figure 6).

In summary, variations in ovarian development between Chahua BCs and EHs were closely related to the number of SYFs, HFs, and plasma hormone levels. Transcriptome sequencing revealed that the extracellular matrix, which is directly related to reproduction, was enriched. FSH, PRL, and ESR might participate in the mechanisms orchestrating extracellular matrix turnover mediated by MMP13 in the Chahua hen ovary, regulating ovarian development and ovulation, and affecting broodiness. These DEGs may be involved in the hormonal regulation of ovarian follicular development and ovulation, thereby affecting broodiness of Chahua chickens. In conclusion, these results not only provide a theoretical basis for research on the regulatory mechanism of egg production, but also identify candidate genes for genetic improvement.

Supplementary materials

Supplementary data are available in Supplementary Files.

Acknowledgments

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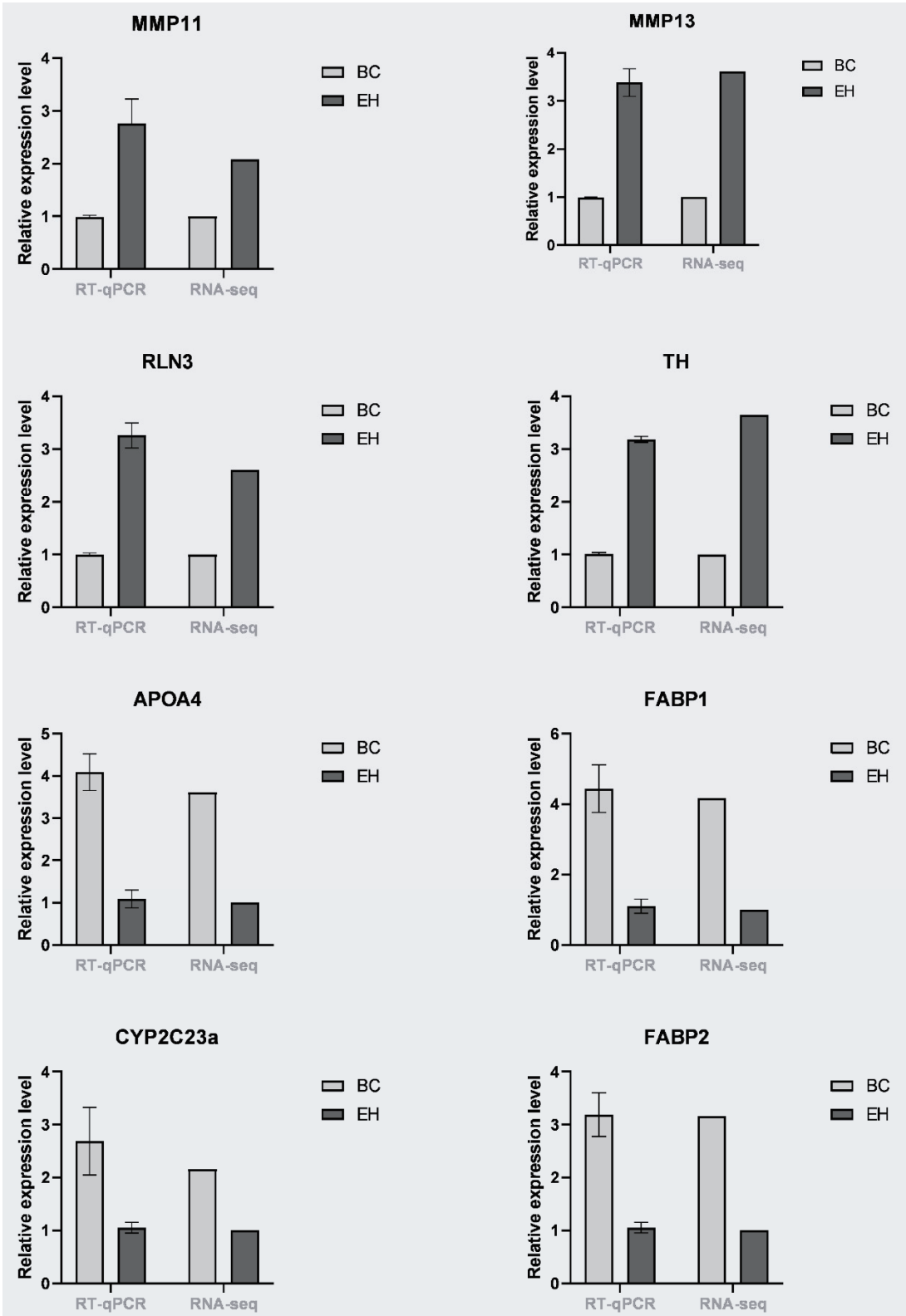

Fig. 4. **Validation of differentially expressed genes (DEGs) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).**

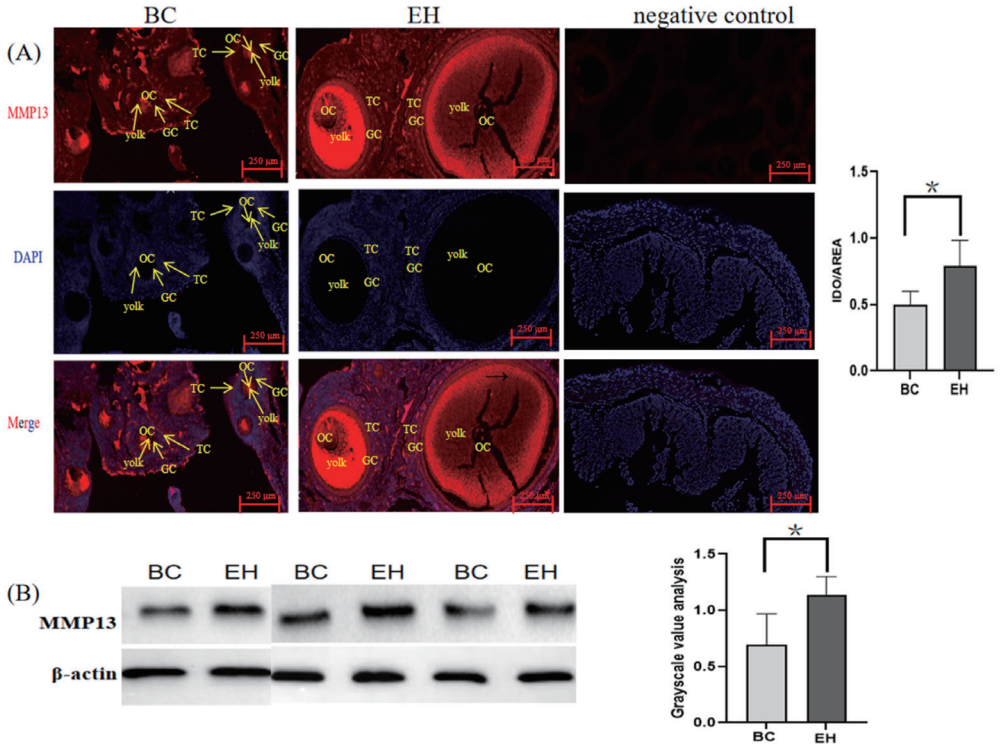

Fig. 5. **Validation of matrix metalloproteinase 13 (MMP13) by immunofluorescence (IF) (A) and western blotting (WB) (B).** BCs, broody chickens; EHs, egg-laying hens; OC, oocyte; GC, granular cell; TC, theca cell.

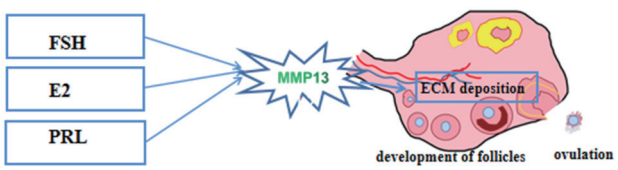

Fig. 6. **Summary of possible mechanisms that cause development of follicles and ovulation in the ovary.** Follicle-stimulating hormone (FSH), prolactin (PRL), and estrogen (E_2) may participate in the mechanisms orchestrating extracellular matrix turnover mediated by matrix metalloproteinase 13 (MMP13) in the Chahua hen ovary, regulating ovarian development, ovulation, and affecting chicken broodiness. ECM, extracellular matrix.

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Author Contributions

Yanli Du and Meiquan Li designed the experiments; Jinshan Ran and Wei Huang analyzed the data and wrote the manuscript; Xiao Wang and Cuilian Wang performed the experiments; Jing Wang and Na Zhu raised the animals.

Conflicts of Interest

The authors declare no conflict of interest.

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