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Transcriptome analysis of ovary in relatively greater and lesser egg producing Jinghai Yellow Chicken

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ABSTRACT

Egg production is determined by the function of ovary and is regulated by the hypothalamic-pituitary-ovary axis. The mechanism by which the ovary regulates egg production, however, is still poorly understood. The purpose of this study is to compare the transcriptome difference in ovary of relatively greater and lesser egg producing chickens, and to screen candidate genes related to egg production. A RNA sequencing was performed to analyze and compare the mRNA in ovarian tissues of relatively greater and lesser egg producing chickens. A total of 4 431 new genes expressed in the chicken ovary were mined. There were 305 differentially expressed genes (DEGs) identified between the relatively greater and lesser egg producing hens. Gene ontology analysis identified five candidate genes related to egg production, including *ZP2*, *WNT4*, *AMH*, *IGF1*, and *CYP17A1* genes. Tissue expression profiles indicated these five candidate genes were highly expressed in chicken ovarian tissues, indicating a potential role in regulating chicken ovarian function and egg production. The KEGG analysis indicated the neuroactive ligand-receptor interaction pathway might have an important function in regulation of egg production. In addition, four known pathways related to reproduction were detected, including the calcium signaling, wnt signaling pathway, focal adhesion, and cytokine-cytokine receptor interaction pathways. Results of the present study indicate gene expression differences in the ovarian tissues of relatively greater and lesser egg producing chickens, and identified five important candidate genes related to egg production, which provided a theoretical basis for improving egg production of Jinghai Yellow Chickens.

1. Introduction

Egg production is an important trait of chickens, which affects the production efficiency and profit of the laying hen industry. Egg production traits are determined by ovarian function and is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which secretes specific neuropeptides or hormones to stimulate oocyte maturation and ovulation (Kang et al., 2012). The regulation of ovarian function has been the subject of research for several decades. Early studies focused on the regulation of steroidogenesis in the

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ovary and it is now well established that the granulosa and theca cell layers of the follicles are the source of several steroid hormones (Huang et al., 1979; Wang and Bahr, 1983; Onagbesan and Peddie, 1988). In recent times, there has been more attention on the growth of the follicles until maturation, the incidence of follicular atresia, the transition of follicles between different stages of the follicular hierarchy or cell differentiation and the control of the ovulation process (Onagbesan et al., 2009; Price, 2016; Nowak et al., 2017; Worku et al., 2017; Richani and Gilchrist, 2018).

It is well established that ovarian function is regulated by pituitary gonadotropins (LH and FSH). It is also becoming clearer from recent studies that locally-produced factors such as growth factors, steroids, cytokines, neuropeptides, and adipokines have essential modulatory functions in the regulation of ovarian function by the pituitary hormones (Dupont et al., 2008; Field et al., 2014; Ervin et al., 2017; Kranc et al., 2017; Richani and Gilchrist, 2018).

Results of several studies indicate there are many mRNA transcripts in the hypothalamus and pituitary gland (Shiue et al., 2006; Chen et al., 2007a, 2007b), ovarian follicles (Yang et al., 2008), liver (Ding et al., 2008), and shell glands (Yang et al., 2007) that are associated with egg production variability in chickens. These studies were conducted using the cDNA chip, which is gradually being replaced by RNA sequencing. The RNA sequencing approach has been widely used in gene alternative splicing, skin color, immune, and feed conversion efficiency research in chicken (Zhou et al., 2014; Sevane et al., 2015; Zhang et al., 2015a, 2015b, 2015c).

In the present study, RNA sequencing was conducted to compare ovarian mRNA transcriptomes between relatively greater and lesser egg producing chickens. The aim was to identify candidate genes involved in regulating ovarian function, thereby affecting egg production. There was also a focus on determining biological processes and pathways related to chicken egg production.

2. Materials and methods

This experiment was performed in accordance with Chinese guidelines for animal welfare, and the animal protocol was approved by the animal welfare committee of Yangzhou University.

2.1. Sample preparation and RNA extraction

Eight chickens (four relatively greater egg producing chickens and four relatively lesser egg producing chickens) of similar body weight were selected at 300 days of age. The differences of egg production between the two groups were significant ($P < 0.01$; Supplementary data 1). The lighting program is provided in Supplementary Table 2. The eight chickens were anesthetized with sodium pentobarbital post egg laying and the whole ovarian tissue, which comprised various sizes of follicles (large yellow follicles, > 8 mm diameter; small yellow follicles, 5 to 8 mm diameter; white cortical follicles, 1 to 5 mm diameter) was collected according to the method described by Yang et al. with some modification (Yang et al., 2008; Onagbesan et al., 2009). The total RNA was subsequently isolated from this tissue using Trizol reagent (Invitrogen, Carlsbad, USA). The integrity, concentration and purity of total RNA were assessed using the Nanodrop, Qubit 2.0, and Aglient 2100. The detailed method of sample preparation is reported in Supplementary Table 3.

2.2. Library construction and sequencing

The mRNA was enriched using a magnetic bead with Oligo (dT) and then randomly fragmented using Fragmentation Buffer. The mRNA was used as template to synthesize the first-strand cDNA using random hexamers. The second-strand cDNA was synthesized using buffer (Invitrogen), dNTPs, RNase H, and DNA polymerase I. The products were purified by AMPure XP beads and then the end of the double strand was repaired and A-tailed. Suitable sized fragments were selected using AMPure XP beads to construct the cDNA library by PCR. The RNA sequencing was performed using Illumina HiSequencing 2500.

2.3. Data analysis

The raw data were flirted to remove the adaptor primers and poor quality reads to obtain clean data. The Mapped Data were obtained by mapping the Clean Data to the reference genome Galgal4 (ftp://ftp.ensembl.org/pub/release-75/fasta/gallus_gallus/) using TopHat2 program (Kim et al., 2013). Mapped reads were spliced and compared to the genome annotation information of *Gallus gallus* using the Cufflinks program (Trapnell et al., 2012) to mine new genes. All the new genes were compared with the proteins in Swiss-Prot (Apweiler et al., 2004), GO (Ashburner et al., 2000), COG (Tatusov et al., 2000), and KEGG (Kanehisa et al., 2004) databases.

2.4. Differential expressed gene analysis

The Fragments per Kilobase of transcript per Million Fragments Mapped (FPKM) value was used to measure the abundance of each transcript. Relevance among biological replicates was evaluated using Pearson's Correlation Coefficient r^2 (Schulze et al., 2012). The T04 and T08 samples were rejected from the DEGs analysis due to a low correlation with other samples in the same group. The DEGs were identified using the DEseq program (Anders and Huber, 2010) with Fold Change ≥ 2 and False discovery rate (FDR) < 0.05 being determined.

Table 1
Primers of differentially expressed genes for RT-qPCR.

Primer	Sequence (5'→3')	Accession number
IL9R	F: ACAAGTGTTGGAACGTATGCTT R: TAATGCACAGGTGAAGGCCC	XM_015294508.1
IGF1	F: AAACACTGTGTGGTGCTGAG R: GTGGTGTAAGCGTCTACTGC	NM_001004384.2
PARVA	F: CGCTGGCGAGGAGGAAAAA R: AGCATAGTGTCCTCAGGGTCT	XM_015286436.1
MYLK	F: GGGAAGGAGGAGGAGAACCT R: GACAGAGCTGCGGGGAAC	NM_001322361.1
GABRA1	F: GGACTGGGAGAGCGTGTAAC R: ATCTTTCCAGCTTTGGCGGA	NM_204318.2
LTB4R	F: TAAGTGGCACAGTAGGACCC R: ACCTTGCCTTCCAAGTGGTTT	XM_015292300.1
NTSR1	F: ATCTGCACCACCATCGTGAA R: ACGACCATGGGAAACACGAA	NM_001245982.1
CCR5	F: GTCACCACTTGCACCTTATGTT R: ATAAAAGCTGTGTGCGGCCC	NM_001271141.1
IL15	F: AGTGCAAATTTATCGTAGTTCCGT R: AGAACAGAATCTGGATCGGGAA	NM_204571.1
VIPR2	F: AAGCATGCAAAACCAGTGGAC R: AATCTTTGCAGGACGCCAAC	NM_001014970.1
EDAR	F: GGCCTTGTCTCCCAGGTTAC R: GGTAGCCCCTGCACATTCTT	NM_001012611.1
beta-Actin	F: CAGCCATCTTTCTTGGGTAT R: CTGTGATCTCCTTCTGCATCC	NM_205518.1

2.5. RT-qPCR analysis of DEGs

Eleven candidate genes that were up-regulated in the relatively greater egg producing group were randomly selected to perform RT-qPCR to verify the RNA-sequencing results. The primers of 11 DEGs are shown in Table 1. The *beta-actin* gene was used as the housekeeping gene. The RT-qPCR was performed using the SYBR® Premix Ex Taq™ II kit according to the instruction. The ABI 7500 software (v 1.4) was used to detect the fluorescent signals. Quantification of selected mRNA transcript abundances was performed using the comparative threshold cycle [$2^{-\Delta\Delta Ct}$] method [$\Delta Ct = Ct_{target\ gene}\text{-}Ct_{housekeeping\ gene}$, $\Delta\Delta Ct = \Delta Ct\text{-}\Delta Ct_{mean}$, ΔCt_{mean} is the mean of ΔCt value of the group with largest ΔCt value)].

2.6. Tissue expression pattern of important candidate genes

Tissue relative mRNA transcript abundance pattern of five important candidate genes were determined by RT-qPCR in eight tissues. The primers of five candidate genes are shown in Table 2. The RT-qPCR and qantification of mRNA transcript abundances were performed with the same methods as described in the Materials and methods Section 2.5 of this manuscript.

2.7. Statistical analyses

All data were presented as the mean ± standard deviation. Significant differences between samples were calculated by Student's t-test. All tests were performed at least in triplicate. All statistical analyses were conducted using the SPSS for Windows software (version 22, SPSS, Inc.) and Excel software (Microsoft Corp.). The $P < 0.05$ value was considered to indicate a statistically significant difference.

Table 2
Primers of five important candidate genes for RT-qPCR.

Primer	Sequence (5'→3')	Accession number
ZP2	F: TGATGTCTTGTGAGCACGCTGTAG R: GTGCCATTCAGCAGCAGGAG	NM_001039098.1
CYP17A1	F: GGAGCTGACAGATGACCACC R: TCTTCTGGACCTCGGGGTAG	NM_205030.1
IGF1	F: AAACACTGTGTGGTGCTGAG R: GTGGTGTAAGCGTCTACTGC	NM_001004384.2
WNT4	F: CAGTACCAATTCCGCAACCG R: CCTGCTGAAGAGATGGCGTA	NM_204783.1
AMH	F: AGCGCTTCCTTATCCTGCAC R: ATGGAACGGCAGCCTGAAC	NM_205030.1

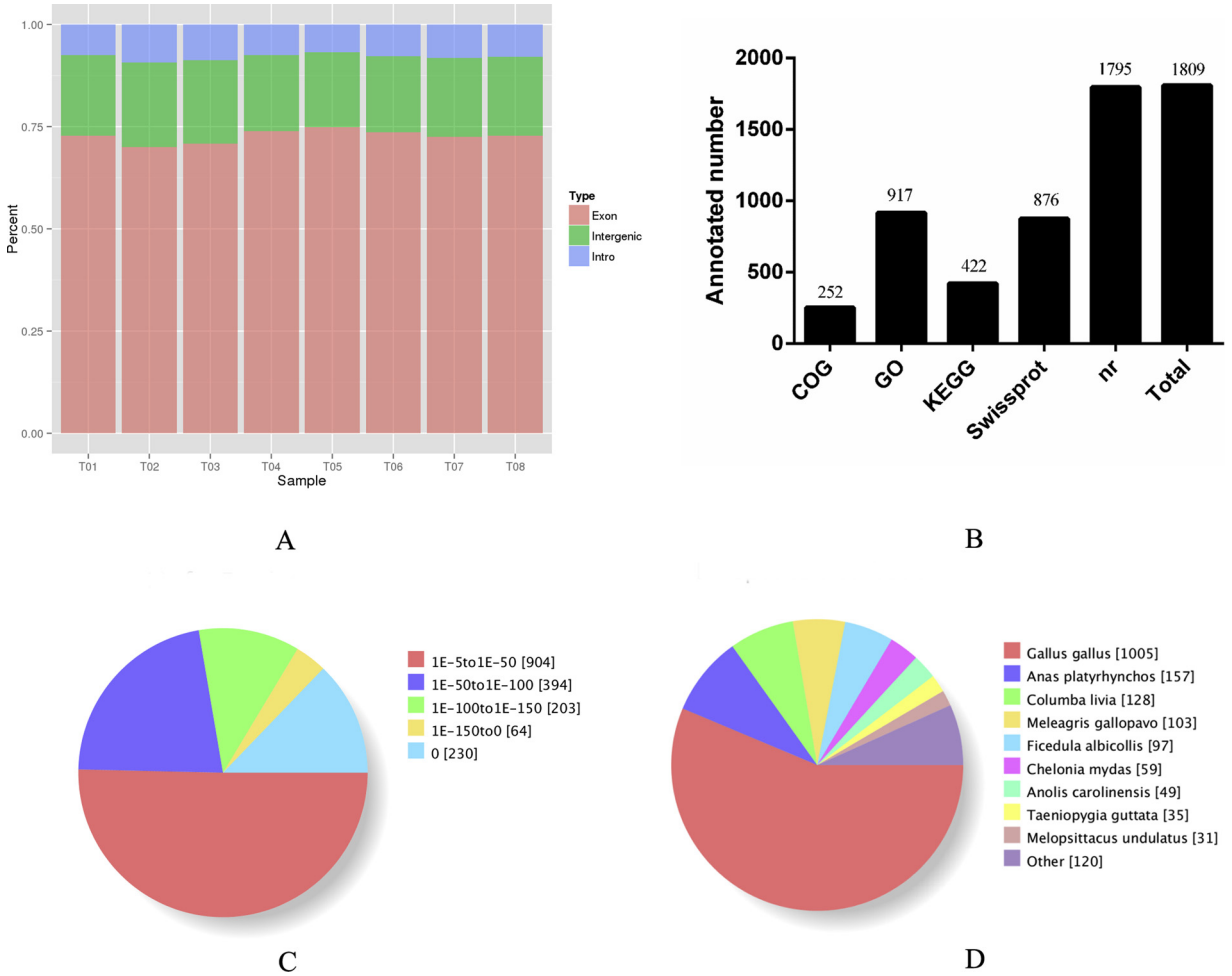

Fig. 1. RNA sequencing results and new gene mining. A: Distribution of mapped reads on exon, intergenic, and intron region; Each column represents one sample: the pink, green, and blue areas represent exon, intergenic, and intron region, respectively; Height of a column represents the percent of reads mapped to each region; Mapped reads in the intergenic and intron region still occupied a certain percentage, indicating that the reference genome needs to be further improved. B: Annotated number of new genes from five databases; C: Distribution of the E value for annotated genes. 99.2% new genes shared significant sequence similarity with mapped sequences in five databases (E ≤ 1e-5); D: Distribution of species match to the annotated genes; More than half (55.6%) of these 1809 annotated genes matched with the *Gallus gallus* sequence, followed by *Anas platyrhynchos* (8.7%), *Columba livia* (7.1%), *Meleagris gallopavo* (5.7%), *Ficedula albicollis* (5.4%), *Chelonia mydas* (3.3%), *Anolis carolinensis* (2.7%), *Taeniopygia guttata* (1.9%), *Melopsittacus undulates* (1.7%) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3. Results

3.1. RNA sequencing results

Eight cDNA libraries of ovaries from Jinghai Yellow Chickens were constructed. There were 61.09GB of clean data, including 484,992,074 reads that were generated after quality control assessment. Clean data were aligned to the reference genome of *Gallus gallus*. The results indicated that 242,496,487 mapped reads with 252 base pair (bp) average length and 52.8% GC content were obtained. The mapped ratio between reads and the reference genome of all the samples ranged from 73.9% to 78.4%. This indicates the reference genome met the requirement for subsequent analysis. The distribution of mapped reads on different regions (exon, intron, and intergenic region) of the reference genome was counted (Fig. 1A). The sequencing data were submitted to the Sequence Read Archive (Run Accession Number SRR4897316) in NCBI.

3.2. New gene mining

The Cufflinks program (v2.2.1) was used to assess unannotated transcripts, mine new genes and thereby enrich and improve the annotation information of the genome. 4,431 new genes were mined and performed to sequence alignment against nr, Swiss-Prot,

GO, COG, and KEGG databases using the BLAST program (Altschul et al., 1990). In total, 1809 new genes were successfully annotated. In Fig. 1B, there is a depiction of the number of genes annotated in each database. There were 1,795 (99.2%) new genes that shared significant sequence similarity with mapped sequences in five databases (E ≤ 1e-5). The E-values of the annotated genes are included in Fig. 1C. More than half (55.6%) of the 1,809 annotated genes matched the *Gallus gallus* sequence, followed by *Anas platyrhynchos* (8.7%), *Columba livia* (7.1%), *Meleagris gallopavo* (5.7%), *Ficedula albicollis* (5.4%), *Chelonia mydas* (3.3%), *Anolis carolinensis* (2.7%), *Taeniopygia guttata* (1.9%), *Melopsittacus undulates* (1.7%) (Fig. 1D).

3.3. GO analysis of new genes

There were 4,431 new genes assessed with GO analysis procedures using the Blast2Go (v3.0) program (Conesa et al., 2005) to predict gene functions. A total of 2,820, 1,139, and 3,781 genes were assigned to the cellular component, molecular function, and biological process GO categories, respectively. The distribution of the annotated new genes as ascertained using the GO analysis are depicted in Fig. 2. In the cellular component category, cell (23%) and cell (22%) part terms were the most abundant terms. In the molecular function category, binding (53%) and catalytic activity (24%) were the most abundant, while in the biological process category, the cellular process (17%) and single-organism process (16%) were the most abundant terms.

3.4. KEGG analysis of new genes

There were 572 new genes annotated and mapped using the KEGG pathways procedures. The five KEGG pathways with the greatest ranking were MAPK signaling pathway, endocytosis, ubiquitin mediated proteolysis, RNA transport, and regulation of actin cytoskeleton. Furthermore, there were many genes that were mapped to oocyte meiosis and GnRH signaling pathways, which are related to the reproduction process. The mapped KEGG pathways are depicted in Fig. 3A.

3.5. COG annotation

To integrate and annotate the sequencing data more efficiently, the new genes were aligned with the COG database. Genes in general function term accounted for the largest proportion, followed by replication, recombination and repair, translation, ribosomal structure and biogenesis, transcription and posttranslational modification, protein turnover, and chaperones (Fig. 3B).

3.6. Differential expressed gene analysis

In the present study, 305 DEGs were identified in the ovaries of the relatively greater and lesser egg producing chickens (Supplementary data 4). Compared with the relatively lesser egg producing group, 145 genes were up-regulated and 160 genes were down-regulated in the relatively greater egg producing group. The MA plots can be useful for determining any systematic bias that may be present between conditions (A = log2 (Fold change); M = -log10 (P value)). A volcano plot was developed to assess the relationship between significance and fold change (Fig. 4).

The results from using GO analysis of DEGs indicated there were 48, 15, and 58 genes assigned to cellular component, molecular function, and biological process categories, respectively (Fig. 5A). In the cellular component category, five terms were enriched: extracellular region, extracellular region part, extracellular space, proteinaceous extracellular matrix, and extracellular matrix categories. In the molecular function category, neurotransmitter receptor activity, neurotransmitter binding, G-protein coupled acetylcholine receptor activity, and muscarinic acetylcholine receptor activity were enriched terms, while in the biological process category, cell surface receptor linked signal transduction, G-protein coupled receptor protein signaling pathway, Wnt receptor signaling pathway, calcium modulating pathway, biological adhesion, and cell adhesion were enriched. Results from use of the KEGG analysis indicated DEGs were enriched in the neuroactive ligand-receptor interaction pathway. The top five enriched KEGG pathways were neuroactive ligand-receptor interaction, hedgehog signaling pathway, cytokine-cytokine receptor interaction, calcium signaling pathway, and melanogenesis (Fig. 5B).

To explore the difference in the function of up-regulated and down-regulated genes, 145 up-regulated genes and 160 down-regulated genes were categorized to the biological process and pathway analysis categories. Biological process results indicated the up-regulated genes were mainly involved in the G-protein coupled receptor protein signaling pathway and cell surface receptor linked signal transduction, while the down-regulated genes were mainly involved in the wnt receptor signaling pathway and the calcium modulating pathway (Fig. 6A). Results from pathway analysis assessments indicated there were down-regulated genes that were enriched in three pathways: hedgehog signaling pathway, melanogenesis, and wnt signaling pathway, while there were no pathways in which there was enrichment for up-regulated genes (Fig. 6B).

3.7. Genes related to egg production

To assess candidate genes related to egg production, DEGs with a FPKM < 1 were filtered out. Genes involved in reproductive processes were selected as candidate genes, including wingless-type MMTV integration site family member 4 (*WNT4*), cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), zona pellucida glycoprotein 2 (*ZP2*), insulin like growth factor 1 (*IGF1*), and anti-Mullerian hormone (*AMH*). There was also identification of several pathways that have been reported to be related to egg production, including the calcium signaling pathway, wnt signaling pathway, Focal adhesion, oocytesis meiosis, and cytokine-cytokine receptor

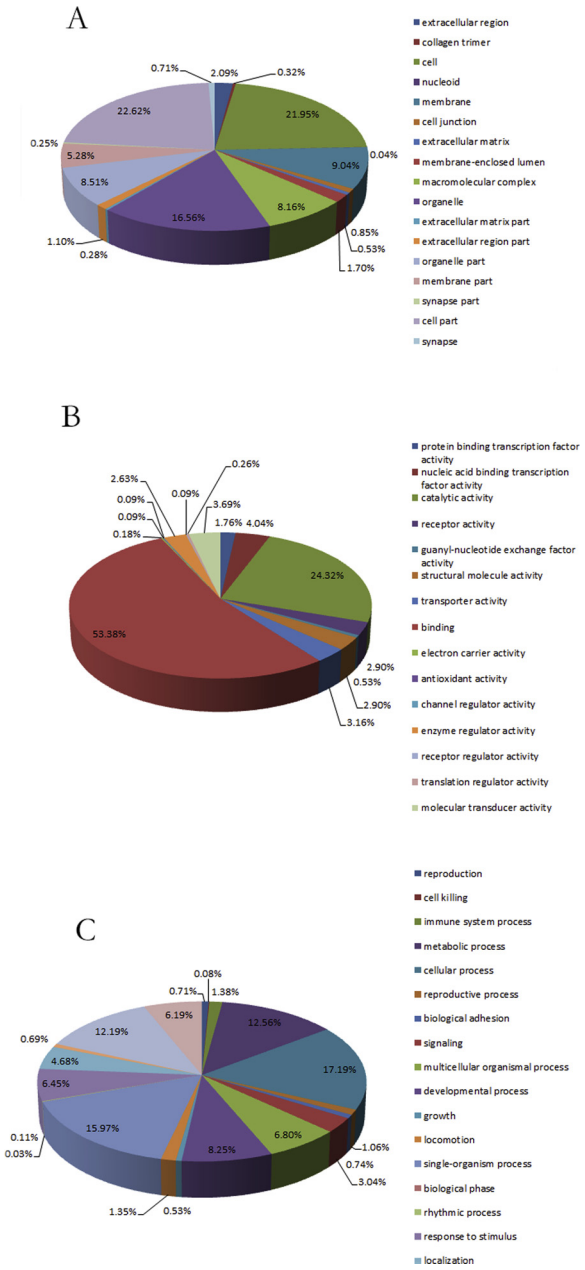

Fig. 2. Distribution of the annotated new genes in three GO categories. A: Cellular component, B: molecular function, C: biological process; In cellular component category, the cell (23%) and cell (22%) part terms were the most abundant terms; In the molecular function category, binding (53%) and (24%) catalytic activity were the most abundant, while in the biological process category, cellular process (17%) and single-organism process (16%) were the most abundant terms.

interaction pathways. The *WNT4*, wingless-type MMTV integration site family, member 6 (*WNT6*), wingless-type MMTV integration site family, member 10A (*WNT10A*), *IGF1*, parvin, alpha (*PARVA*), myosin, light chain kinase (*MYLK*), chemokine (C-C motif) receptor 5 (*CCR5*), interleukin 15 (*IL15*), interleukin 9 receptor (*IL9R*), and ectodysplasin A receptor (*EDAR*) genes were associated with functions in these pathways. Furthermore, neuroactive ligand-receptor interaction was the most enriched pathway, in which seven DEGs were involved including vasoactive intestinal peptide receptor 2 (*VIPR2*), 5-hydroxytryptamine (serotonin) receptor 1B (*HTR1B*), and cholinergic receptor, muscarinic 5 (*CHRM5*), gamma-aminobutyric acid (GABA) A receptor, alpha 1 (*GABRA1*), neurotensin receptor 1 (high affinity) (*NTSR1*), similar to M3 muscarinic acetylcholine receptor; cholinergic receptor, muscarinic 3 (*CHRM3*), and leukotriene B4 receptor (*LTB4R*). In Fig. 7, there is a depiction of the relative gene expression of all these candidate genes for the relatively lesser and greater egg production chickens.

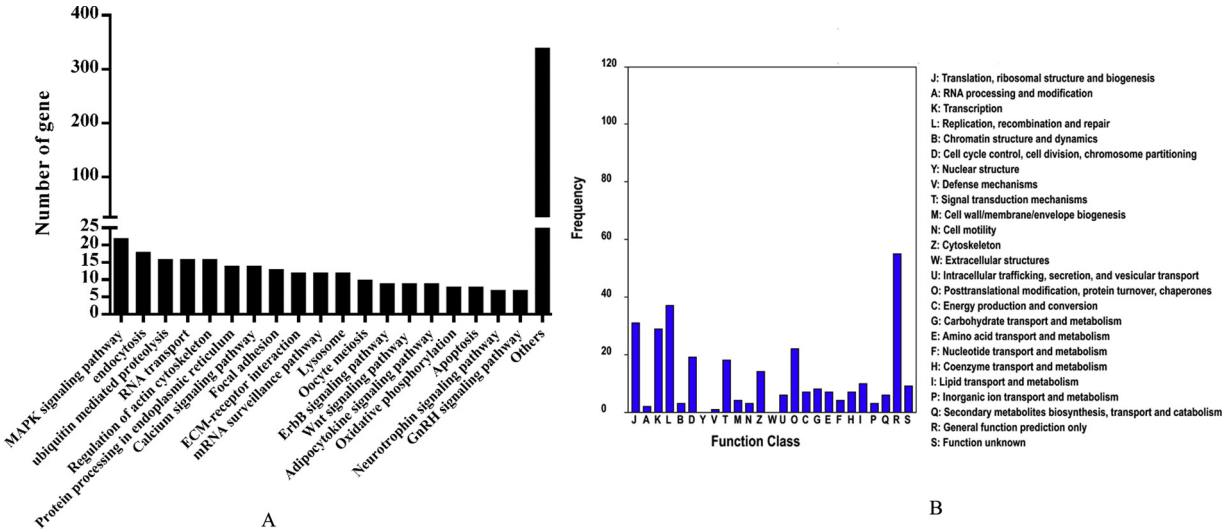

Fig. 3. Pathways and COG function analysis of new genes; A: The top five KEGG pathways were MAPK signaling pathway, endocytosis, Ubiquitin mediated proteolysis, RNA transport, and regulation of actin cytoskeleton; Furthermore, many genes were mapped to oocyte meiosis and GnRH signaling pathway pathways, which were related to reproduction process; B: COG function classification of new genes; Genes in general function term accounted for the largest proportion, followed by replication, recombination and repair, translation, ribosomal structure and biogenesis, transcription and posttranslational modification, protein turnover, chaperones.

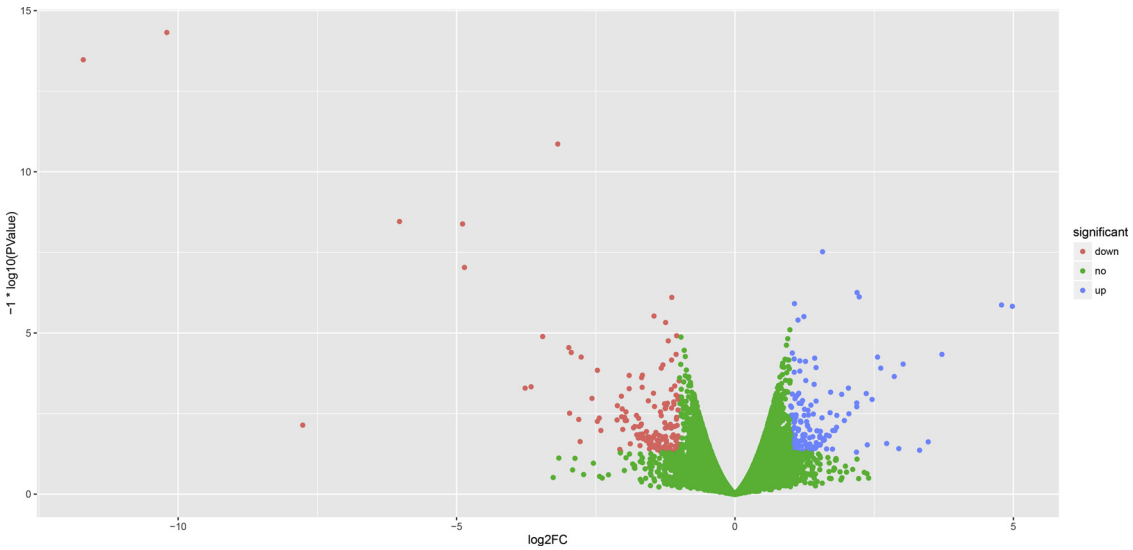

Fig. 4. Volcano plot of all expressed genes; The red plots represent significantly down-regulated genes; the blue plots represent significantly up-regulated genes; the green plot represents genes with no significance (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.8. RT-qPCR validation of DEGs

To confirm the DEG results obtained using RNA-sequencing, RT-qPCR was used to verify the relative abundance of mRNA transcripts of 11 DEGs, which included important genes related to egg production reported in a previous study. The result indicated that there was a consistency in relative abundances of mRNA transcripts for the genes of interest with the RNA-sequencing results (Fig. 8).

3.9. Tissue gene expression pattern of important candidate genes

There was assessment of the relative abundance of mRNA for five important candidate genes, including *ZP2*, *CYP17A1*, *IGF1*, *WNT4*, and *AMH*, in eight chicken tissues using RT-qPCR. The results indicated *ZP2*, *WNT4*, and *AMH* mRNA transcripts were in

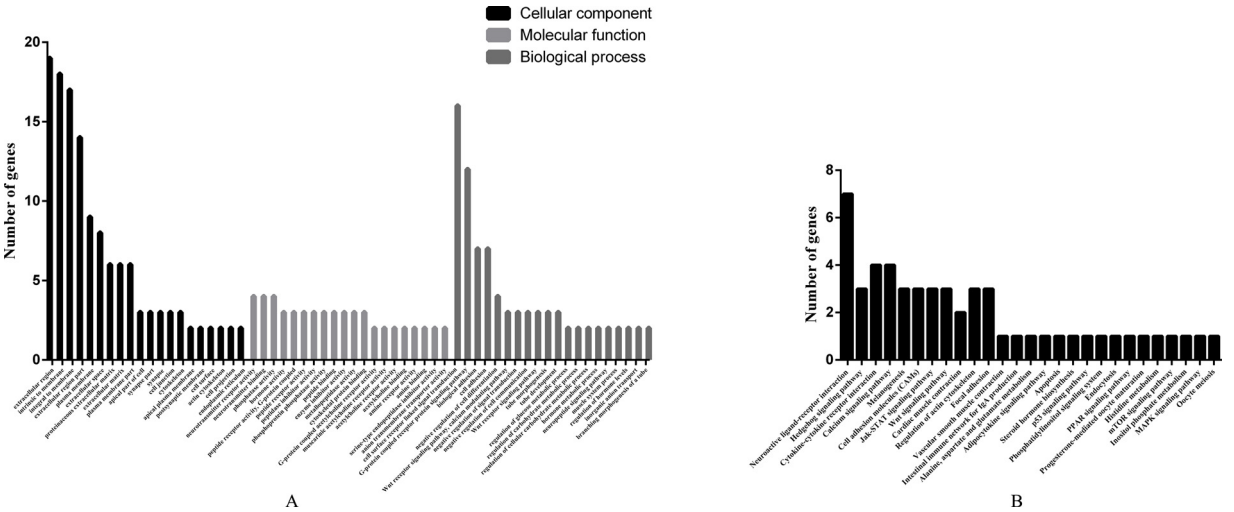

Fig. 5. GO (A) and pathway (B) enrichment analysis of all the DEGs.

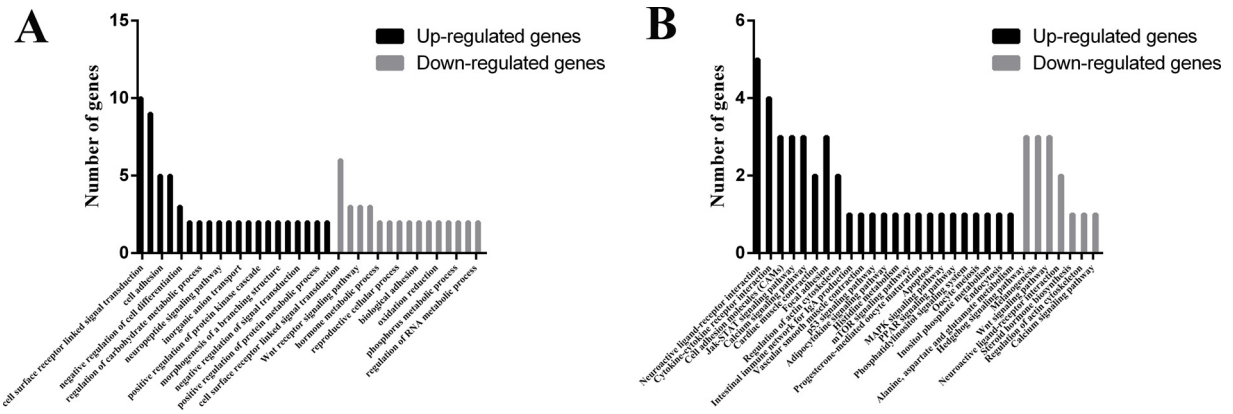

Fig. 6. GO (A) and pathway (B) analysis of significant up and down regulated genes.

greatest relative abundance in ovarian tissues. The *CYP17A1* mRNA transcript was in greatest relative abundance in the liver, ovary, heart, and kidney tissues. The *IGF1* mRNA transcript was in large relative abundance in ovarian and leg muscle tissues (Fig. 9).

4. Discussion

Greater egg production is the aim of modern poultry production. Traditional methods to improve egg production by selecting for egg number or rate of lay for specific time periods has resulted in positive genetic progress (Fairfull and Gowe, 1990). With the rapid development of high-throughput DNA sequencing technology, the methods of animal breeding research have gradually evolved from conventional breeding to a use of a combination of various omics techniques. High-throughput sequencing such as RNA sequencing provides for a platform to study the complex biological functions involving a large number and networks of coded genes when there are specific physiological conditions (Shiue et al., 2006). In poultry, the reproductive endocrine system and the reproductive functions are controlled by the hypothalamic-pituitary-gonadal axis (Padmanabhan et al., 2002). Consequently, the ovary is an important tissue in which to search for candidate genes associated with egg production, because genes may be expressed differentially in different physiological conditions for egg laying.

In the present study, there were detailed mRNA profiles obtained for ovarian tissues of hens from a relatively greater and lesser egg producing lines. To our knowledge, this is the first report of mRNA profiles associated with gene expression profiles in the ovaries of hens from these relatively greater and lesser egg producing hens using RNA sequencing. There were 305 DEGs identified in ovaries of the relatively greater and lesser egg producing hens. The GO analysis results indicated there were five genes involved in reproductive processes, including *ZP2*, *WNT4 AMH*, *IGF1*, and *CYP17A1* genes. Results from tissue expression profiles indicated all five of these genes above were highly expressed in ovarian tissues, indicating the likely association in regulation of functions of the chicken ovary.

Chicken *ZP2* is expressed in immature small ovarian follicles and remains as an egg-coat component locally in the germinal disc region of mature eggs. This protein may have a function in the preferential binding and penetration of sperm in the germinal disc

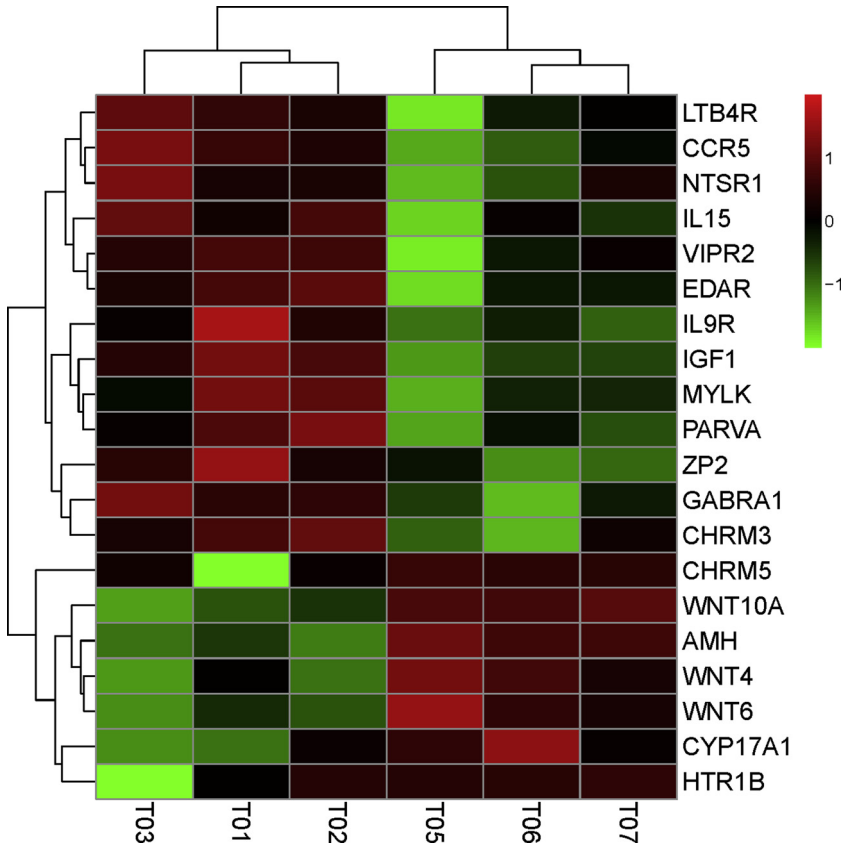

Fig. 7. Clustering heatmap of genes related to egg production. T01, T02, and T03 represent samples from hens with relatively greater egg production, while T05, T06, and T07 represent samples with relatively lesser egg production Color, ranging from green through black to red, indicates relatively greater and lesser gene expression (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

region of eggs (Nishio et al., 2014). The WNT4 protein is one of a few factors with a function in the ovarian-determination pathway, likely having important functions in development and function of the oviduct as well as initiation of ovarian carcinogenesis in laying hens (Lim et al., 2013). The AMH protein might have a function in ovarian follicular development in chickens. The abundance of *AMH* mRNA is maximal in growing follicles to approximately 6–8 mm in size and is markedly less around the time of follicle selection. This finding indicates there may be an important function for AMH in regulating the pre-selection pool of ovarian follicles (Johnson, 2012). There is evidence that IGF1 protein is involved in the regulation of progesterone production by granulosa cells, and androgen and estrogen production by theca cells of the avian ovary (Tosca et al., 2008). The IGF1 protein is required for the prevention of follicle atresia and there is an increased probability of the follicle progressing through the pre-ovulatory stage to ovulation as a result of actions of IGF1 (Li et al., 1999; Onagbesan et al., 2009). The CYP17A1 is an important enzyme in conversion of pregnenolone exclusively to 17-hydroxypregnenolone and also regulates the cleavage of the C17–C20 carbon-carbon bond of 17-hydroxypregnenolone to form dehydroepiandrosterone via Δ^5 pathway (Yoshimoto and Auchus, 2015). In hens, the *CYP17A1* gene is expressed mainly in the theca interna, and may regulate the synthesis of estradiol and testosterone in the chicken ovary (Kato et al., 1995). Although these results of these previous studies indicate that these five genes might have important functions in regulating the egg production of chickens, there needs to be more focused studies to ascertain the functions of these genes in egg production in the future.

The results of the KEGG analysis of DEGs in the present study indicate that the neuroactive ligand-receptor interaction was the most important pathway contributing to the differential egg production between the relatively greater and lesser producing hens. Results of a previous study indicate the neuroactive ligand-receptor interaction in the ovarian tissues was also enriched in a comparative transcriptomic analysis of relatively greater and lesser egg production ducks (Tao et al., 2017). Seven DEGs of *VIPR2*, *HTR1B*, *CHRM5*, *GABRA1*, *NTSR1*, *CHRM3*, and*LTB4R* were involved in this pathway. All of these genes are reported for the first time as a result of the present study to contribute in the regulation of ovarian function in chickens.

Results of previous studies indicate the functions of the calcium signaling pathway, wnt signaling pathway, focal adhesion and cytokine-cytokine receptor interaction pathway were related to egg production and eggshell quality in poultry (Chen et al., 2007a, 2007b; Zhang et al., 2015a, 2015b, 2015c; Tao et al., 2017). Likewise, in the present study, results indicated these pathways were enriched and ten DEGs were involved, indicating that these pathways may have functions in regulating chicken ovarian function. The

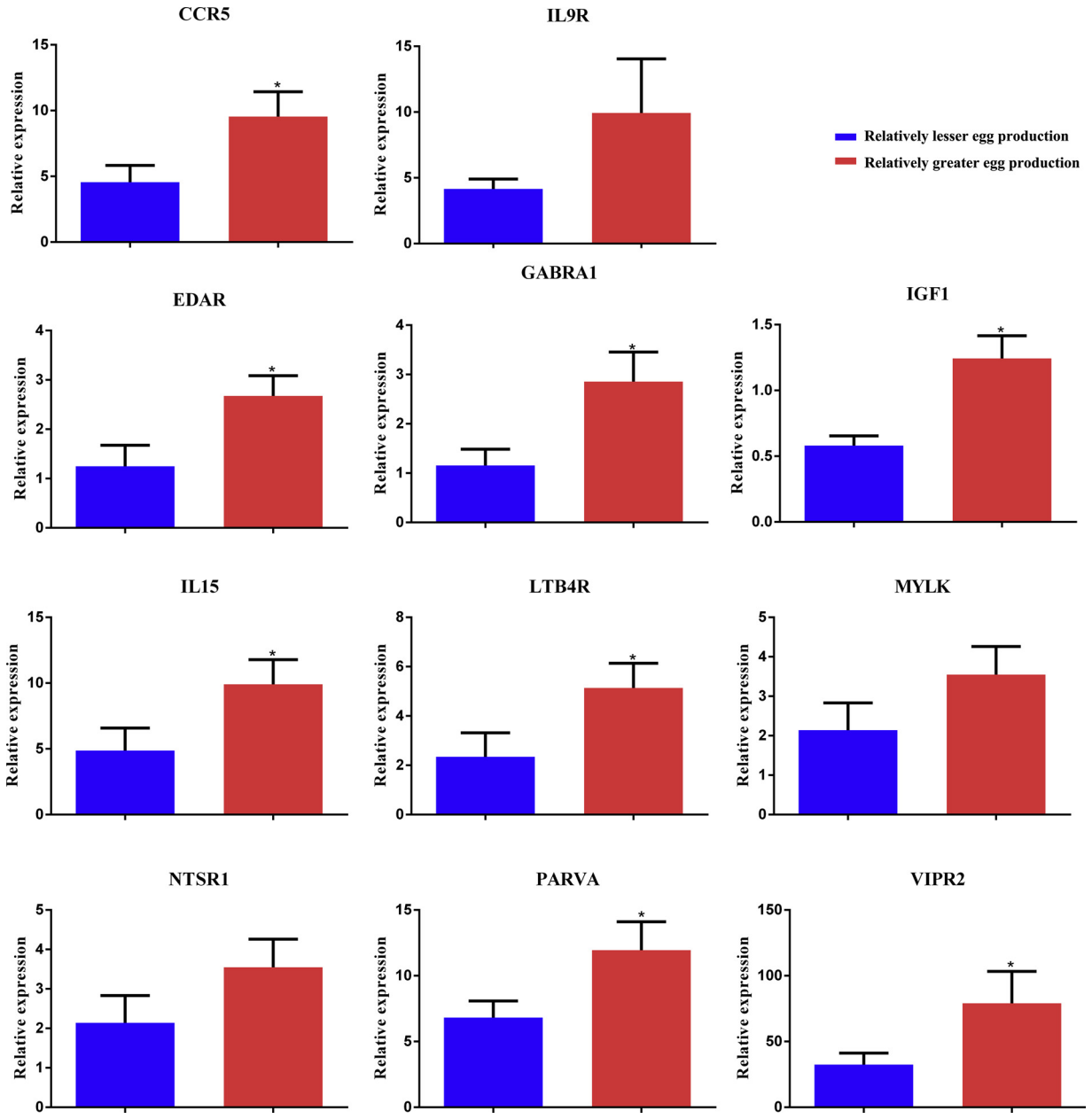

Fig. 8. Validation of important genes related to egg production by RT-qPCR; Blue and red represent relatively lesser and greater egg production group, respectively; * indicates the relative expression is different between relatively lesser and greater egg producing chickens ($P < 0.05$); Quantification of selected gene expression was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

activation of Wnt signaling inhibits FSH target gene transcription and steroid production associated with maturation and differentiation of the ovarian follicle in rats (Stapp et al., 2014). In the present study, three down-regulated genes, *WNT4*, *WNT6*, and *WNT10A*, were enriched in this pathway. This finding indicates the wnt signaling pathway may have an inhibitory action in the regulation of chicken ovarian functions. The seven genes enriched in calcium signaling pathway, focal adhesion and cytokine-cytokine receptor interaction pathway were all up-regulated in the relatively greater as compared with lesser egg producing group, indicating that these three pathways may have a positive effect on chicken egg production in chickens. The *MYLK* and *PARVA* genes were expressed to the greatest (FPKM > 10) extent of the seven up-regulated genes. These two genes, therefore, might have relatively more important function in the regulation of chicken egg production.

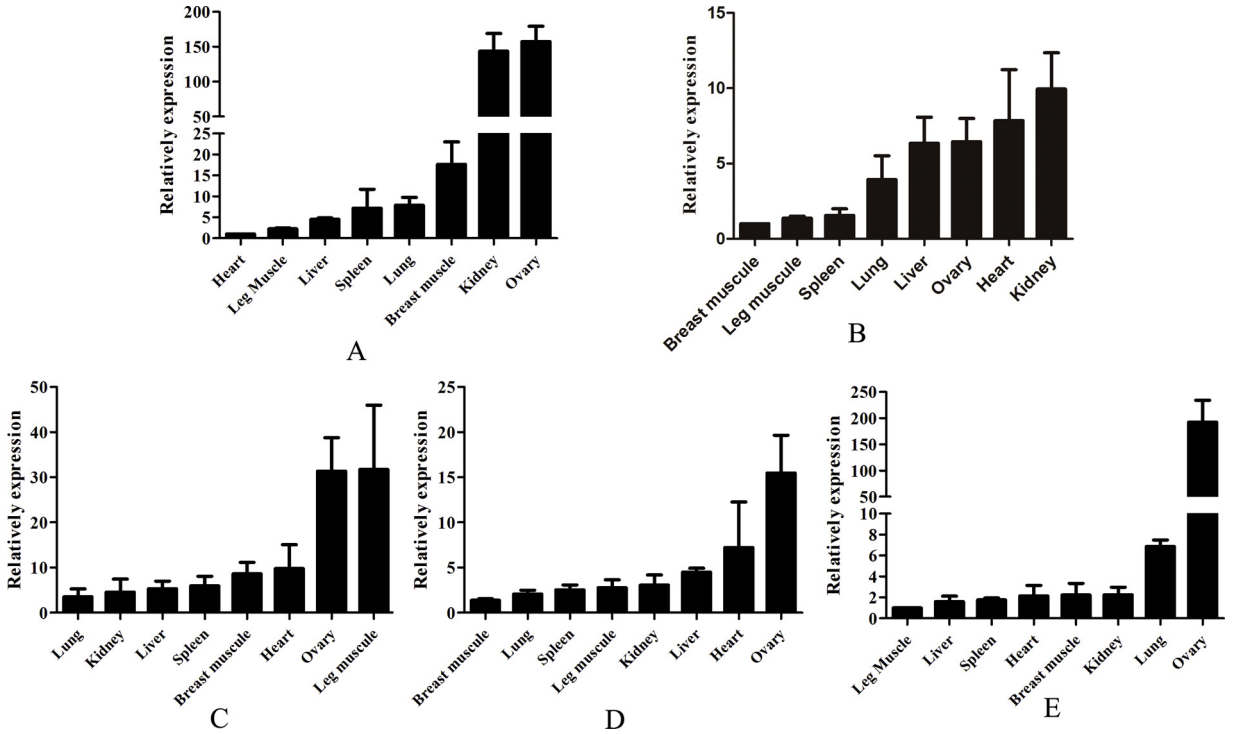

Fig. 9. Tissue expression pattern of five candidate genes. A: *ZP2* gene; B: *CYP17A1* gene; C: *IGF1* gene; D: *WNT4* gene; E: *AMH* gene; Results indicate *ZP2*, *WNT4*, and *AMH* genes were expressed to the greatest extent in ovarian tissues; *CYP17A1* gene was highly expressed in liver, ovary, heart, and kidney tissues; *IGF1* gene was highly expressed in ovary and leg muscle tissues.

5. Conclusion

In conclusion, the present study is an original report of the transcriptome analysis of ovaries from the relatively greater and lesser egg producing Jinghai Yellow Chickens. The results provide a global view of the chicken ovarian transcriptome. Most importantly, 305 differentially expressed genes were identified for the two egg production groups. The results not only provide a theoretical basis for regulatory mechanism research of egg production, but also provide candidate genes for genetic improvement of egg production.

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Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.106114.

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