Transcriptome comparative analysis of ovarian follicles reveals the key genes and signaling pathways implicated in hen egg production

Xue Sun¹,²†, Xiaoxia Chen¹,²†, Jinghua Zhao¹,², Chang Ma¹,², Chunchi Yan¹,², Simushi Liswaniso¹,², Rifu Xu¹,²* and Ning Qin¹,²*

Abstract

Background: Ovarian follicle development plays an important role in determination of poultry egg production. The follicles at the various developmental stages possess their own distinct molecular genetic characteristics and have different biological roles in chicken ovary development and function. In the each stage, several genes of follicle-specific expression and biological pathways are involved in the vary-sized follicular development and physiological events. Identification of the pivotal genes and signaling pathways that control the follicular development is helpful for understanding their exact regulatory functions and molecular mechanisms underlying egg-laying traits of laying hens.

Results: The comparative mRNA transcriptomic analysis of ovarian follicles at three key developmental stages including slow growing white follicles (GWF), small yellow follicles (SYF) of recruitment into the hierarchy, and differentiated large yellow follicles (LYF), was accomplished in the layers with lower and higher egg production. Totally, 137, 447, and 229 of up-regulated differentially expressed genes (DEGs), and 99, 97, and 157 of down-regulated DEGs in the GWF, SYF and LYF follicles, including VIPR1, VIPR2, ADRB2, and HSD17B1 were identified, respectively. Moreover, NDUFA81 and GABRA1 genes, two most promising candidates potentially associated with egg-laying performance were screened out from the 13 co-expressed DEGs in the GWF, SYF and LYF samples. We further investigated the biological effects of NDUFA81 and GABRA1 on ovarian follicular development and found that NDUFA81 promotes follicle development by stimulating granulosa cell (GC) proliferation and decreasing cell apoptosis, increases the expression of CCND1 and BCL-2 but attenuates the expression of caspase-3, and facilitates steroidogenesis by enhancing the expression of STAR and CYP11A1. In contrast, GABRA1 inhibits GC proliferation and stimulates cell apoptosis, decreases the expression of CCND1, BCL-2, STAR, and CYP11A1 but elevates the expression of caspase-3. Furthermore, the three crucial signaling pathways such as PPAR signaling pathway, cAMP signaling pathway and neuroactive ligand-receptor interaction were significantly enriched, which may play essential roles in ovarian follicle growth, differentiation, follicle selection, and maturation.

© The Author(s) 2021. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Conclusions: The current study provided new molecular data for insight into the regulatory mechanism underlying ovarian follicle development associated with egg production in chicken.

Keywords: Ovarian follicle, Transcriptome, NDUFA81, GABRA1, Egg production

Background
Egg production initiates from the follicle prehierarchical and hierarchical development, maturation, and finally ovulates from the hen ovary, which is governed by the hypothalamic–pituitary–ovarian axis [1–3]. Ovarian follicle development plays a key role in the egg production capacity, which is characterized by a well-organized follicular hierarchy in high production egg-laying layers. In chickens with low egg-laying rates, e.g., the broiler breeder hen, follicular development is not well-organized, which leads to reduced productivity [1]. Generally, ovarian follicles can be categorized by size or/and according to color (white or yellow), into at least four types, including small white resting follicles (less than 2 mm diameter), slow growing white follicles (GWF, from 2 mm up to 6 mm diameter), small yellow follicles (SYF, 6 up to 8 mm) of recruitment into the follicular hierarchy (at the stage of follicle selection), and large yellow follicles (LWF) at the differentiated preovulatory stage, being 9 to 12 mm in diameter and five to six hierarchical follicles of increased sizes (from F6 to F1) in hen ovary [4, 5]. In the different phases of follicular development, many divergent biological processes affect oocyte growth, and proliferation and differentiation of granulosa and theca cells within the various-sized follicles [4, 6, 7]. Moreover, a plethora of ovarian paracrine and autocrine factors was involved in regulation of the follicle development and its function as well as the positive or negative controls through the endocrinal hormones from the hypothalamus and pituitary, including gonadotropin releasing hormone (GNRH), gonadotropin inhibitory hormone (GNIH), and follicle stimulating hormone (FSH) [1, 2, 8]. Within the ovary, the most representative hormones and growth factors such as steroidogenic-related enzymes steroidogenic acute regulatory protein (STAR), hydroxysteroid (17beta) dehydrogenase 1 (HSD17B1) and cytochrome P450 side-chain cleavage (P450scC/CYP11A1), intra-ovarian hormones progesterone (P4), estradiol (E2) and anti-müllerian hormone (AMH), cell proliferation or apoptosis-related factors Bcl-2, cyclin D1 (CCND1) and caspase-3 (CASP3), which have indispensable effects on follicular development, follicle selection or atresia, finally on their preovulatory development and ovulation, making the early developmental differences of ovarian follicles and egg production capacity in adult layers highly correlated, have been intensively investigated [9–13]. Undoubtedly, follicular size has a close relationship with its developmental biology, physiological function, and molecular regulation. It has been drawing so much attention to extensive exploration of the key genes and signaling pathways to be implicated in ovarian follicle development and potentially associated with egg production in chicken.

To intensively explore the molecular regulatory mechanisms underlying follicle development for better understanding egg production capacity, previous studies on transcriptome analyses of ovarian follicles, granulosa cells and theca cells of follicles as well as hypothalamus, pituitary gland and ovary, have revealed many genes involved in ovarian follicular development and follicle selection, which potentially correlate with the high capacity for egg production in chicken, goose, duck, and turkey. It has been reported that 20 differentially expressed transcripts (e.g., FTH1, TB, EEF1A1, TXN, ANXA2, ING4, and ACADL) associated with high rates of egg production in chicken ovarian follicles were screened out by cDNA microarray data analysis [14]. Several differentially expressed transcripts implicated in steroidogenesis (STAR, HSD3B, CYP11A1, and CYP19), paracrine signaling (PCSK6, KITL and WNT4) and transcription (FOXO3, FOXL2 and WT1) were identified by transcriptome analysis of the small ovarian follicles 0.5, 1, and 2 mm in diameter after oocyte removal in chicken, which may be important during early avian follicular development [15]. Transcriptomic analysis of single small yellow follicles demonstrated that Wnt4 takes part in the regulation of chicken follicle selection [16]. Transcriptomic analysis found 855 candidates differentially expressed between small yellow follicles (SYF, 6–8 mm in diameter) and F6 follicles in laying hens, including VLDLR1, WIF1, NGFR, AMH, BMP15, GDF6 and MMP13. They might play certain roles in chicken follicle selection [17]. Integrated transcriptomic analysis on chicken SYF differing in follicle stimulating hormone (FSH) receptor expression discovered 467 differentially expressed genes (DEGs). And sosondowah ankyrin repeat domain family member A (SOWAH) gene was confirmed to affect the expression of genes involved in chicken follicle selection and to inhibit the proliferation of granulosa cells [18]. Moreover, RNA sequencing was used to analyze mRNAs and long noncoding RNAs (lncRNAs) from granulosa cells of SYFs from Jinghai Yellow chickens in exposure to red light and white light groups. One thousand one hundred eighty-two DEGs were identified and the integrated network...
analysis shown that several of them were involved in follicular development through steroid hormone synthesis, oocyte meiosis, and the PI3K-Akt signaling pathway [19]. Additionally, similar studies on transcriptome analysis of ovarian follicles were also reported in goose, duck and turkey. It was published that transcriptomic profiling identifies 688 DEGs in Huoyan goose ovaries (including the small and large yellow follicles) between the laying period and ceased period, in which the 12 validated genes, i.e., PGR, INSR, NPY1R, ESRRB, MEL1C, VIPR2, LHCGR, SCG2, GHR, STAR, HSD3B2 and CYP11A1, are mainly involved in the signal transduction pathways for reproduction regulation, such as steroid hormone biosynthesis, GnRH signaling pathways, oocyte meiosis, progesterone-mediated oocyte maturation, steroid biosynthesis, calcium signaling pathways, and G-protein coupled receptor signaling pathway [20]. Ren showed seven key DEGs, including EPOX, STAR, CYP17, 3β-HSD, CYP1B1, CYP19A1, and SR-B1, were related to Peking duck ovarian follicle development [21]. Moreover, transcriptome analysis of the granulosa layer of the largest follicle (F1) and fifth largest follicle (F5), theca interna layer of F5 follicle, and small white follicle samples from low and high egg producing turkey hens revealed 12,221 DEGs were identified, and among them, several of the most promising candidates and signaling pathways may be mainly involved in differential regulation of the hypothalamo-pituitary-thyroid axis, particularly thyroid hormone transporters and thyroid hormone receptors, and of estradiol signaling [22]. However, the pivotal genes and signaling pathways controlling the development of various-sized ovarian follicles in chicken, and their exact physiological roles and regulatory mechanisms in egg production remain largely unclear yet.

The Jilin Black (JB) chicken, an indigenous Chinese breed (dual-purpose type for egg and meat production), is characterized by high quality meat, but a low rate of egg production, low laying peak, and strong broodiness [23]. In contrast, the Lohmann Brown (LB) layer, a commercial egg-laying breed, is characterized as possessing a high rate of egg production, high laying peak, and great consistency in laying performance [24]. The differences in the egg production capacity between the JB and LB breeds may, therefore, serve as a proper basis for discovering potential key genes involved in egg production and for further investigating the molecular mechanism underlying formation of egg-laying traits in chicken.

To find the most potential genes and signaling pathways associated with egg production, comparative transcriptome analysis of ovarian GWF, SYF and LYF follicles between the JB and LB breeds was implemented. The present study aims to identify key functional genes implicated in ovarian follicular growth and development (including follicle selection) that directly contribute to egg-laying production. These results laid a molecular foundation for further elucidating the regulatory mechanisms of the promising genes in ovary development, and the pivotal signaling pathways in formation of chicken egg-laying traits.

**Results**

**Comparison of egg-laying production related traits between JB and LB hens**

Performance of egg-laying production related traits in JB and LB hens was evaluated, including age at first egg (AF), average body weight at 21 and 66 weeks of age (21BW and 66 BW), egg weight at 66 weeks of age (EW), hen-housed egg production (HH) at 72 weeks of age, and egg production (total egg numbers per hen) rate at 21, 30, 43, 57 and 66 weeks of age (21EN, 30EN, 43EN, 57EN and 66EN). As shown in Table 1, the AF, which is the days of age when egg production rate per day reaches 50%, was significantly earlier in LB than JB hens ($P<0.001$). HH at 72 weeks of age was higher in LB than JB hens ($P<0.001$). Moreover, egg production rates at 21, 30, 43, 57 and 66 weeks of age were also higher in LB when compared to JB hens ($P<0.001$).

**Characteristics of RNA sequencing data**

Nine cDNA libraries of the GWF, SYF and LYF samples from JB and LB layers were constructed, respectively. In total, 822,883,832 sequence reads were obtained, and with an average of 45,430,781 clean reads each sample (Supplementary Table S1). On average, the clean data of high quality accounted for 99.09% of the sequences of raw reads, 85.59% of the clean reads were mapped to the reference genome of *gallus gallus* and 81.97% reads were mapped uniquely. The dynamic range of the expression

### Table 1 Comparison of egg productions between JB and LB hens

| Breed | AF  (day) | 21BW | 66BW | EW  | HH  | 21EN | 30EN | 43EN | 57EN | 66EN |
|-------|----------|------|------|-----|-----|------|------|------|------|------|
| LB(n=720) | 141.2* | 1.71±0.13 | 1.98±0.45 | 64.7±2.26 | 289.6* | 53.49* | 93.82* | 88.56* | 83.75* | 74.34* |
| JB(n=458) | 206.5 | 1.78±0.19 | 2.21±0.76 | 62.9±4.48 | 152.7 | 11.32 | 54.62 | 64.39 | 57.18 | 46.27 |

*AF* age at first egg (days), *BW* average body weight (kg) at 21 and 66 weeks of age, *EW* average egg weight (g) at 66 weeks of age, *HH* average hen-housed egg production (egg numbers) at 72 weeks of age, *EN* average egg production rate (%) at 21, 30, 43, 57, and 66 weeks of age. The superscript asterisks indicate significant difference of the means at the level, $P<0.001$.
values was estimated and exhibited as a box plot of logarithmic transformed FPKM values for each sample separately (Fig. 1A), and the FPKM density distribution is presented in Fig. 1B.

Identification of differentially expressed genes and signaling pathway enrichment

In this study, differentially expressed genes (DEGs) in the follicles of GWF, SYF and LYF between JB and LB hens were identified (The relevant datasets have been published by our group and are available at PRJNA669967 and PRJNA670553 in NCBI), respectively. In GWF follicles, 137 up-regulated and 99 down-regulated DEGs were revealed by comparison of JB with LB chickens (Fig. 2A; |log2FoldChange| > 1, P < 0.05). Among them, the most important potential genes causing development of the undifferentiated follicles, which may be associated with egg production performance, were uncovered such as GABRA1, ADGRD1, VIPR2, APOD, BRINP1, PAK5, GPR65, RYR2, ZBPB, PIK3R1, EDIL3, TP53I3, BLK, POSTN, PERP1, HACD4, ZPI, WNT2B, CYP4F22, ADRB2, STARD4, PRLL, NCAM2, WISP1, MC2R, LOC424014, and RXRG (Table 2). Moreover, the significant top 20 signaling pathways were displayed (Fig. 2B), including PPAR signaling pathway, neuroactive ligand-receptor interaction, cAMP signaling pathway, fatty acid biosynthesis, cell adhesion molecules (CAMs), and AMPK signaling pathway.

In SYF follicles, 447 of up-regulated and 97 of down-regulated DEGs were discovered in comparison of JB with LB chickens (Fig. 3A). Among them, the most interesting candidate DEGs to be implicated in follicle selection and differentiation, including GTSF1, P2RX1, GABRA1, GABRB2, NGF, ADGRD1, ADCY7, SRGN, SOX9, BCL2A1, GPR65, BCL2L14, ALDH1A1, BLK, GABRG1, METRN, P2RY12, CYP1C1, ADRB2, TENM2, GPR157, NDUFB1, STMN3, PRL, SOCS2, TLE4Z1, NCAM2, DTDH1, LOC424014, CYP2AB3, and HSD17B1 were found (Table 3). Furthermore, the significant top 20 signaling pathways were identified (Fig. 3B). Among them, only two of the pathways, NF-kappa B signaling pathway, and CAMs, may be much closely linked with follicle development.

In LYF follicles, 229 of up-regulated and 157 of down-regulated DEGs were mined from JB and LB chickens (Fig. 4A). Of which, the most promising DEGs to be involved in follicle growth, differentiation and maturation such as APCDD1, GABRA1, TSHB, APOBEC2, PRL, GHRHR-LR, NPY4R, BMPR2, TRH, HPGD, LEPR, CYP2D6, VIP, GREB1, SPEF2, PERP1, CRH, MAP3K9, RGS22, FABP7, SSTR2, RBP3, NDUFB1, STMN3, PRL, GAP43, NCAM2, LOC770492, CDKN1A, ID4, LOC424014, and CYP2AC2 were unveiled (Table 4). Moreover, the significant top 20 signaling pathways were obtained (Fig. 4B), which included retrograde endocannabinoid signaling, PPAR signaling pathway, neuroactive ligand-receptor...
interaction, mucin type O-glycan biosynthesis, JAK-STAT signaling pathway, circadian entrainment, CAMs, and cAMP signaling pathway.

To reveal the most important DEGs and signaling pathways potentially involved in follicle development and differentiation across the three developmental stages GWF, SYF and LYF, the NDUFAB1 and GABRA1 genes, two most promising candidates potentially associated with egg-laying performance were screened out from the 13 co-expressed DEGs in the GWF, SYF and LYF samples. The three crucial signaling pathways, including PPAR signaling pathway (ko 03320), cAMP signaling pathway (ko 04024), and neuroactive ligand-receptor interaction (ko 04080) were significantly enriched. Moreover, NDUFAB1 gene as a member of oxidative phosphorylation pathway (gga: 00190; Supplementary Fig. S1) was involved in the PPAR signaling pathway; while GABRA1 gene as a component of neuroactive ligand-receptor interaction pathway (gga: 04080; Supplementary Fig. S2) was implicated in the G protein-coupled receptor pathway (gga: 04030) which contains the FSH/FSHR signaling pathway, and in the cAMP signaling pathway.

Validation of the selected DEGs by RT-qPCR
Based on the analyses aforementioned, 20 representatives of most relevant DGEs, i.e., VIPR2, GABRA1, PERP1, ZP1, WISP1, MC2R, STARD4 and NDUFAB1 that differentially expressed in GWF follicles, BCL2L14, LOC424014, ADRB2, GABRA1, PRL, HSD17B1, NCAM2 and NDUFAB1 in SYF follicles, CYP2D6, CRH, GABRA1, GHRHR-LR, ID4, SSTR2, CDKN1A and NDUFAB1 in LYF follicles between JB and LB hens were selected for validation by RT-qPCR. The results showed the significantly differential expression levels of the most transcripts determined by RT-qPCR analysis are consonant with the observation detected by RNA-seq (Fig. 5). While there are significant differences in the expression levels of the other genes, i.e., GABRA1, PERP1, BCL2L14, GHRHR-LR, MC2R, STARD4, NDUFAB1, PRL, and NCAM2, the observed expression trends were concordant with the RNA-seq results, confirming the transcriptome sequencing data are reliable, and can be further investigated for understanding the genetic mechanism of ovarian follicle development associated with egg production in chicken.
Confirmation of the roles of \textit{NDUFAB1} and \textit{GABRA1} genes in ovarian follicle development

To further confirm the exact roles of \textit{NDUFAB1} and \textit{GABRA1} genes in ovarian follicle development, silence of the genes was performed by infection of the shRNA lentivirus in the GCs from 6 to 8 mm follicles of hen ovaries, respectively. The result demonstrated that in the \textit{NDUFAB1} silencing GCs, expression levels of \textit{STAR}, \textit{CYP11A1}, \textit{CCND1}, and \textit{BCL-2} proteins as well as all of their transcripts were significantly downregulated ($P < 0.01$, Fig. 6; Supplementary Fig. S3), but the expression \textit{Caspase-3} and its transcript were remarkably upregulated ($P < 0.001$). Simultaneously, cell proliferation ratio of the GCs was notably decreased as compared to the negative control ($P < 0.05$, Fig. 7), whereas cell apoptosis rate was markedly increased ($P < 0.001$). It was suggested that \textit{NDUFAB1} promotes follicle development by stimulating GC proliferation and decreasing cell apoptosis, increases the expression of \textit{CCND1} and \textit{BCL-2} but attenuates the expression of \textit{caspase-3}, and facilitates steroidogenesis by enhancing the expression of \textit{STAR} and \textit{CYP11A1}.

For \textit{GABRA1} silence of GCs, the expression levels of \textit{STAR}, \textit{CYP11A1}, \textit{CCND1}, and \textit{BCL-2} transcripts and their proteins were notably elevated ($P < 0.01$, Fig. 8; Supplementary Fig. S4). Oppositely, the expression \textit{CASP3} transcript and its protein were significantly reduced ($P < 0.001$). Furthermore, cell proliferation ratio of the GCs was remarkably enhanced as compared to the negative control ($P < 0.01$, Fig. 9), conversely, cell apoptosis rate was sharply decreased ($P < 0.001$). It was proposed that \textit{GABRA1} inhibits GC proliferation and

| Name     | Description                                           | Regulation | Chr. | Log$_{FC}$ | $P$-value     |
|----------|-------------------------------------------------------|------------|------|------------|---------------|
| GABRA1   | gamma-aminobutyric acid type A receptor alpha 1 subunit | up         | 13   | 2.2084     | 4.849E-07     |
| ADGRD1   | adhesion G protein-coupled receptor D1                | up         | 15   | 1.6618     | 0.0364        |
| VIPR2    | vasoactive intestinal peptide receptor 2              | up         | 2    | 2.2675     | 1.7403E-07    |
| APOD     | apolipoprotein D                                      | up         | 9    | 2.1226     | 3.8281E-05    |
| BRINP1   | BMP/retninoic acid inducible neural specific 1         | up         | 17   | 2.1930     | 0.0195        |
| PAK5     | p21 (RAC1) activated kinase 5                         | up         | 3    | 1.0703     | 0.0047        |
| GPR65    | G protein-coupled receptor 65                         | up         | 5    | 1.5475     | 0.0103        |
| RYR2     | ryanodine receptor 2                                  | up         | 3    | 3.2383     | 0.0048        |
| ZPBP     | zona pellucida binding protein                        | up         | 2    | 3.0925     | 0.0042        |
| PIAK3R1  | phosphoinositide-3-kinase regulatory subunit 1        | up         | Z    | 1.9585     | 0.0419        |
| EDIL3    | EGF like repeats and discoidin domains 3              | up         | Z    | 2.0187     | 0.0052        |
| TPS3L3   | tumor protein p53 inducible protein 3                 | up         | 3    | 2.3365     | 0.0311        |
| BLK      | BLK proto-oncogene, Src family tyrosine kinase        | up         | 3    | 1.2293     | 0.0085        |
| POSTN    | peristin                                             | up         | 1    | 1.0831     | 0.0325        |
| PERP1    | PERP1, TP53 apoptosis effector                        | up         | 3    | 5.1568     | 5.0997E-05    |
| HACD4    | 3-hydroxyacyl-CoA dehydratase 4                       | up         | Z    | 2.8745     | 0.0171        |
| ZP1      | zona pellucida glycoprotein 1                         | up         | 5    | 1.2576     | 0.0337        |
| WNT2B    | Wnt family member 2B                                 | up         | 26   | 2.5831     | 0.0003        |
| CYP4F22  | Cytochrome P450 family 4 subfamily member 22         | up         | 30   | 1.5370     | 0.0018        |
| ADRB2    | adrenocortico beta 2                                 | up         | 13   | 1.5427     | 0.0292        |
| STAR04   | STAR related lipid transfer domain containing 4      | down       | Z    | -1.3823    | 3.0970E-09    |
| CREG3L   | cAMP responsive element binding protein 3 like 3     | down       | 28   | -1.8905    | 0.0031        |
| ACSB2G2  | acyl-CoA synthetase bubblegum family member 2        | down       | 28   | -1.0163    | 1.6471E-05    |
| GPER1    | G protein-coupled estrogen receptor 1                 | up         | 14   | -1.9229    | 0.0171        |
| NDUFA1B  | NADH:ubiquinone oxidoreductase subunit AB1            | down       | 14   | -1.2524    | 0.0062        |
| NCAM2    | neural cell adhesion molecule 2                      | down       | 1    | -1.9101    | 0.0049        |
| WISP1    | WNT1 inducible signaling pathway protein 1            | down       | 2    | -1.2337    | 4.4686E-06    |
| MC2R     | melanocortin 2 receptor                              | down       | 2    | -2.7876    | 4.9574E-05    |
| LOC424014| putative methyltransferase DDB_G0268948              | down       | 7    | -3.5398    | 5.0597E-20    |
| RXRG     | retinoid X receptor gamma                            | down       | 8    | -1.4435    | 0.0017        |
increases cell apoptosis, decreases the expression of CCND1, BCL-2, STAR, and CYP11A1 but elevates the expression of caspase-3.

Discussion
Ovary is an important reproductive organ in adult poultry, which contains many various-sized follicles corresponding to the different follicular developmental stages, including the small white follicle resting (months or years) stage (< 2 mm), the slow growing undifferentiated prehierarchical stage (white, 2–6 mm), the stage of follicle selection (small, yellow; 6–8 mm), and the final differentiated hierarchy (large, yellow) stage [1, 4, 5]. The follicles mainly composed of oocytes, granulosa cell layers (GCs) and theca cell layers, are the major compartments which enable the ovary to execute its dual function of gametogenesis and steroidogenesis in chicken [25]; its development is a highly intricate and coordinated hierarchical process involving a multitude of biological events controlled by reproductive hormones in the ovary [8, 26, 27]. Undoubtedly, the follicles at the various developmental stages possess their own distinct molecular genetic characteristics and play different roles in contributing to ovary growth and development. Particularly, genetic regulation of 6–8 mm diameter follicles is generally involved in follicle selection [2, 28, 29] and may possess an exclusive influence on hierarchy of undifferentiated prehierarchical follicles. However, the major genes controlling the follicle development at each stage and their exact physiological mechanisms that regulate follicular growth and order of the ovarian follicle hierarchy remain largely unknown.

To mine the key genes implicated in follicular development at the stages of follicle selection, before and after the selection, in this study, transcriptome analysis of the GWF, SYF and LYF follicles was implemented between the JB and LB chicken breeds. To our knowledge, this is one of the few studies to reveal potential pivotal genes of involvement in hen ovarian follicles at the developmental stages immediately before and after follicle selection, which may be associated with high and low rates of egg production. Firstly, in the GWF follicles of this study, the JB hens with low egg production showed higher mRNA levels of VIPR2, GABRA1, PERP1, and ZP1, and lower mRNA levels of WISP1, MC2R, STARD4, and NDU-FAB1 genes, in which the most representative gene VIPR2 is also named pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (VPAC2), encoding the VPAC2/vasoactive intestinal peptide (VIP) receptor (VIPR2) belonging to the VIP/PACAP type II receptors, including VPAC1/VIPR1 and VPAC2/VIPR2 [30, 31]. Previous studies have proved that PACAP, a bioactive peptide transiently expressed in preovulatory follicles, stimulates various ovarian functions, such as intracellular cyclic AMP (cAMP) accumulation, plasminogen activator production, and inhibits GC apoptosis by interacting
with its receptors, PACAP type I receptor (PAC1) and the type II receptors in rat [32–34]. Moreover, PACAP stimulates oocyte growth but block its maturation in early follicles by acting directly on the oocyte via PAC1 and VPAC2 receptors, whose expression is dominant in growth phase [35]. The participation of VIP in ovarian steroids production was confirmed to induce polycystic ovary [36, 37]. Therefore, it was proposed that the higher expression of VIPR2 transcript in GWF developmental stage may be relevant to broodiness and lower egg-laying trait of JB breed by blocking follicle growth and maturation in ovarian follicle development.

In this study, transcriptomic analysis of SYF follicles revealed higher mRNA levels of BCL2L14, LOC424014, ADRB2, and GABRA1, and lower mRNA levels of PRLL, HSD17B1, NCAM2, and NDUFAB1 genes in the JB hens as compared with LB hens, in which the most representative genes, ADRB2 and HSD17B1, have been intensively studied in ovarian functions [38, 39]. The β2 adrenergic receptor (ADRB2) encoded by ADRB2 gene was mainly expressed in the GCs and Theca cells (TCs) of ovarian follicles and regulated the levels of cAMP and steroid production through activation of ADRB2/cAMP/protein kinase A (PKA) signaling pathway and/or ADRB2/cAMP/protein, phospholipase C (PLC)/protein kinase C (PKC)/cAMP response element-binding protein (CREB) signaling cascade [40–42]. However, the excessive ovarian steroid response to gonadotropins and beta-adrenergic stimulation enhanced polycystic ovary syndrome (PCOS), an endocrine disorder characterized

| Name         | Description                          | Regulation | Chr. | Log2 FC | P-value  |
|--------------|--------------------------------------|------------|------|---------|----------|
| GTSF1        | gametocyte specific factor 1          | up         | 27   | 1.0872  | 0.0009   |
| P2RX1        | purinergic receptor P2X 1             | up         | 19   | 1.7095  | 0.0383   |
| GABRA1       | gamma-aminobutyric acid type A receptor alpha 1 subunit | up         | 13   | 1.7586  | 1.2116E-05|
| GABRB2       | gamma-aminobutyric acid type A receptor beta2 subunit | up         | 13   | 1.5317  | 0.0028   |
| NGF          | nerve growth factor                   | up         | 26   | 1.7033  | 0.0481   |
| ADGRD1       | adhesion G protein-coupled receptor D1 | up         | 15   | 2.6266  | 0.0187   |
| ADCY7        | adenylylate cyclase 7                 | up         | 11   | 1.0016  | 0.0289   |
| P2RX7        | purinergic receptor P2X 7             | up         | 15   | 1.6691  | 4.0602E-05|
| SRGN         | serglycin                            | up         | 6    | 1.9189  | 0.0205   |
| SOX9         | SRY (sex determining region Y)-box 9 | up         | 18   | 1.5868  | 0.0084   |
| BCL2A1       | BCL2 related protein A1               | up         | 10   | 1.6446  | 0.0340   |
| GPR65        | G protein-coupled receptor 65         | up         | 5    | 1.3260  | 0.0187   |
| BCL2L14      | BCL2 like 14                         | up         | 1    | 4.3212  | 1.7558E-05|
| ALDH1A1      | aldehyde dehydrogenase 1 family member A1 | up         | 2    | 2.3347  | 0.0099   |
| BLK          | BLK proto-oncogene, Src family tyrosine kinase | up         | 3    | 1.3837  | 0.0034   |
| GABRG1       | gamma-aminobutyric acid type A receptor gamma1 subunit | up         | 4    | 1.8060  | 0.0005   |
| METRN        | meteinor, glial cell differentiation regulator | up         | 14   | 1.0096  | 0.0087   |
| P2RY12       | purinergic receptor P2Y12             | up         | 9    | 1.5714  | 0.0255   |
| LOC424014    | putative methyltransferase DDB_G0268948 | up         | 7    | 2.0292E-06 | 2.3334 |
| CYP1C1       | cytochrome P450 family 1 subfamily C polypeptide 1 | up         | 5    | 2.8918  | 0.0053   |
| ADRB2        | adrenoceptor beta 2                   | up         | 13   | 1.2618  | 0.0388   |
| TENM2        | teneurin transmembrane protein 2      | down       | 13   | −1.4428 | 1.0434E-05|
| GPR157       | G protein-coupled receptor 157        | down       | 21   | −1.14768| 0.0104   |
| NDUFAB1      | NADH: ubiquinone oxidoreductase subunit AB1 | down       | 14   | −1.0438 | 0.0180   |
| STMN3        | stathmin 3                           | down       | 20   | −1.5957 | 0.0110   |
| PRLL         | prolactin-like protein                | down       | 1    | −1.5757 | 0.0130   |
| SOCS2        | suppressor of cytokine signaling 2    | down       | 1    | −1.2871 | 0.0062   |
| TLE4Z1       | transducin like enhancer of split 4-Z1 | down       | 2    | −1.0419 | 0.0164   |
| NCAM2        | neural cell adhesion molecule 2       | down       | 1    | −6.8712 | 0.0013   |
| DTHD1        | death domain containing 1             | down       | 4    | −2.1469 | 3.5106E-05|
| CYP2AB3      | cytochrome P450, family 2, subfamily AB, polypeptide 3 | down       | 9    | −1.6150 | 0.0142   |
| HSD17B1      | Hydroxyl steroid (17-beta) dehydrogenase 1 | down       | 27   | −1.6963 | 0.0243   |
by anovulation, hyperandrogenism and polycystic ovaries [36, 37, 43, 44]. The much abundant expression levels of \( ADRB2 \) gene may induce layer broodiness by activation of adenylate cyclase through the action of G proteins and stimulate anovulation [37, 43].

The hydroxysteroid (17beta) dehydrogenase type 1 (HSD17B1) is a steroidogenic enzyme encoded by \( HSD17B1 \) gene, to efficiently catalyze reversible interconversion of a low-active precursor estrogen estrone (E1) to the highly active E2 that is necessary for normal ovary development [13, 45]. It is the major isozyme in the granulosa cells of the ovary and has a central role in regulating the circulating estradiol concentration as well as its local production in estrogen target cells, locally promotes development, differentiation, and maturation of the follicle [46–48]. Nevertheless, inhibition of HSD17B1 impairs the synthesis of 17β-estradiol, and attenuates action of the estradiol [47, 49], which can directly block ovarian follicle development. Furthermore, HSD17B1 plays a crucial role in controlling cell proliferation and in the regulation of the growth and function of organs [50]. It was suggested that the lower expression levels of HSD17B1 transcript in SYF follicles of JB hens may affect ovarian dominant follicle selection and follicle growth and function by repressing 17β-estradiol production and follicle cell proliferation, and finally lead to a low egg production.

Transcriptomic analysis of LYF follicles revealed higher mRNA levels of \( CYP2D6, CRH, GABRA1, \) and \( GHRHR-LR \), and lower mRNA levels of \( ID4, SSTR2, CDKN1A \) and \( NDUFA81 \) genes in the JB than in the LB layers. Among them, the most representative gene \( GHRHR-LR \), also named \( VIPR1 \), its encoding product VIPR1 was mainly expressed in granulosa cells and residual ovarian tissue [51]. PACAP may promote oocyte maturation in the maturation phase via VPAC1-R on the follicle cells, whose expression surges in full-grown follicles prior to maturation and is consistently high in the follicles undergoing final maturation [35]. Moreover, the genetic polymorphisms of \( VIP \) and \( VIPR1 \) genes were associated with chicken broodiness and egg production [52, 53]. It was intimated that the higher expression levels of \( VIPR1 \) transcript in LYF follicles of JB hens may inhibit ovarian follicle growth, differentiation and maturation, and contribute to the lower egg production.

Interestingly, the significantly up-regulated \( GABRA1 \) mRNA and down-regulated \( NDUFA81 \) mRNA of the GWF, SYF and LYF follicles were co-expressed differentially in JB hen ovaries when compared with LB hen. Previous studies have reported that \( GABRA1 \) gene encodes γ-aminobutyric acid type A receptor α1 subunit (GABRA1) is one subtype of the neurotransmitter γ-aminobutyric acid (GABA) activating GABA_A receptors that act as the components of the gamma-aminobutyric acid signaling pathway [54, 55]. The heteropentameric receptors are formed from an array of subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ and ρ1–3), producing receptors with diverse functional and pharmacological properties [56–58]. The GABA_A receptors are
vital for controlling neuronal excitability and can display significant levels of constitutive activity that contributes to tonic inhibition [58]. It has been established that the progesterone metabolite allopregnanolone, a potent modulator of the GABAA receptor, is sedative in high concentrations but may precipitate paradoxical adverse effects on mood at levels corresponding to luteal phase concentrations in susceptible women [59]. It indicated that GABAA receptors may be loosely linked to reproductive performance in chicken. Furthermore, the configuration of GABRA1 proves the receptor’s vital role in apoptosis regulations, mitochondrial function, inhibitory effects of different neurotransmitters, and synaptic plasticity by interaction with neuroactive steroids such as androstane and progesterone derivatives [57, 60].

Moreover, another studies on NDUFA1 gene demonstrated that the NDUFA1 gene encodes the acyl carrier protein (ACP), NDUFA1 subunit of mitochondrial complex I (NADH: ubiquinone oxidoreductase), which is involved in cytosolic and mitochondrial fatty acid synthesis pathways [61]. ACP molecule is a universal and highly conserved carrier of acyl intermediates during fatty acid synthesis [62], exists either in the cytosol as a separate domain within a large multifunctional fatty acid synthase polyprotein (type I FAS) or in mitochondria as a discrete entity ACP of the bacterial type II FAS [63, 64]. However, the exact roles of GABRA1 and the NDUFA1 in ovarian follicular function remain unclear in chicken.

To further confirm the roles of GABRA1 and NDUFA1 genes in ovarian follicle development, silencing

### Table 4: Characterization of partial DEGs in LYF follicles of JB and LB chickens

| Name        | Description                                           | Regulation | Chr. | Log2FC | P-value  |
|-------------|-------------------------------------------------------|------------|------|--------|----------|
| APCD1       | APC down-regulated 1                                   | up         | 2    | 1.4852 | 0.0052   |
| GABRA1      | gamma-aminobutyric acid type A receptor alpha 1 subunit| up         | 13   | 1.8192 | 1.0197E-06 |
| TSHB        | thyroid stimulating hormone beta                       | up         | 26   | 2.8128 | 0.0113   |
| APOBEC2     | apolipoprotein B mRNA editing enzyme catalytic subunit 2| up         | 26   | 1.0499 | 0.0021   |
| PRLR        | prolactin receptor                                     | up         | Z    | 2.1874 | 8.712E-07 |
| GHRHR-LR    | growth-hormone releasing hormone-like peptide receptor  | up         | 2    | 3.7088 | 0.0236   |
| NPY4R       | neuropeptide Y receptor Y4                             | up         | 6    | 4.0431 | 0.0204   |
| BMP2R       | bone morphogenetic protein receptor type 2             | up         | 7    | 1.0301 | 0.0253   |
| TRH         | thyrotropin releasing hormone                          | up         | 12   | 1.6478 | 0.0474   |
| HPGD        | 15-hydroxyprostaglandin dehydrogenase                  | up         | 4    | 1.4899 | 0.0168   |
| LEPR        | leptin receptor                                        | up         | 8    | 1.1635 | 0.0002   |
| CYP2D6      | cytochrome P450 family 2 subfamily D member 6         | up         | 1    | 1.5767 | 0.0030   |
| VIP         | vasoactive intestinal peptide                         | up         | 3    | 2.4115 | 0.0174   |
| GREB1       | growth regulating estrogen receptor binding 1          | up         | 3    | 1.0473 | 0.0006   |
| SFEF2       | sperm flagellar 2                                      | up         | Z    | 1.3693 | 0.0018   |
| PRRP1       | TP53 apoptosis effector                                | up         | 3    | 4.2193 | 6.265E-06 |
| CRH         | corticotropin releasing hormone                        | up         | 2    | 4.5714 | 1.444E-07 |
| MAP3K9      | mitogen-activated protein kinase kinase kinase 9       | up         | 5    | 1.0666 | 0.0074   |
| ROS22       | regulator of G protein signaling 22                   | up         | 2    | 1.1938 | 0.0013   |
| FABP7       | fatty acid binding protein 7                           | up         | 3    | 1.0730 | 0.0004   |
| SSTR2       | somatostatin receptor 2                                | down       | 18   | -1.6523| 0.0161   |
| RBP3        | retinol binding protein 3                              | down       | 6    | -2.8177| 0.0003   |
| NDUFA1      | NADH: ubiquinone oxidoreductase subunit A1             | down       | 14   | -2.2040| 1.993E-10 |
| STMN3       | stathmin 3                                            | down       | 20   | -1.2830| 0.0434   |
| PRLR        | prolactin-like protein                                 | down       | 1    | -1.4488| 0.0323   |
| GAP43       | growth associated protein 43                           | down       | 1    | -2.7814| 0.0468   |
| NCAM2       | neural cell adhesion molecule 2                       | down       | 1    | -2.0656| 0.0087   |
| LOC770492   | ras-related and estrogen-regulated growth inhibitor-like| down      | 5    | -1.0108| 0.0180   |
| CDKN1A      | cyclin dependent kinase inhibitor 1A                   | down       | 26   | -1.0877| 0.0151   |
| ID4         | inhibitor of DNA binding 4, HLH protein                | down       | 2    | -1.2727| 6.007E-05 |
| LOC424014   | putative methyltransferase DDB_G0268048                | down       | 7    | -1.5789| 6.301E-05 |
| CYP2AC2     | cytochrome P450, family 2, subfamily AC, polypeptide 2| down       | 3    | -3.8372| 0.0022   |
GABRA1 and NDUFAB1 genes were conducted in the GCs of SYF, respectively. The result showed that expression levels of the steroidogenic-related genes, both STAR and CYP11A1, were remarkably enhanced when silencing GABRA1 was implemented in the GCs. As known, the STAR and P450scc were two key enzymes in regulation of P4 synthesis and secretion within the GCs. In which, STAR functions to transport cholesterol to the inner membrane of mitochondria from the outer membrane [65], then P450scc catalyzes transformation of cholesterol to pregnenolone, a P4 precursor [66]. It is well shown that as follicle was recruited into the follicular hierarchy (follicle selection), the granulosa cells initiate differentiation, and accompanied by the P4 synthesis and secretion [67, 68]. P4 is required for growth and ovulation of preovulatory follicles, exerts a stage-dependent role in ovarian follicle development [69]. However, high level of progesterone markedly inhibited ovulation of preovulatory follicles, and generally delayed oviposition by negative feedback fashion on the gonadotroph in hens [69, 70]. In this study, the higher expressions of GABRA1 can decrease P4 production by inhibiting STAR.

Fig. 5 Evaluation and comparison of mRNA expression levels (Log2FC) of 20 candidate genes when analyzed by RNA Seq and quantitative RT-qPCR. 
A The candidates in the differentially expressed in GWF follicles between JB and LB hens. B The candidates in SYF follicles. C The candidates in LYF follicles. The relative expression level of each gene was quantified by the $2^{-\Delta\Delta CT}$ method and 18S rRNA was used as the internal control for normalization of the results.

- **A** The candidates in the differentially expressed in GWF follicles between JB and LB hens.
- **B** The candidates in SYF follicles.
- **C** The candidates in LYF follicles. The relative expression level of each gene was quantified by the $2^{-\Delta\Delta CT}$ method and 18S rRNA was used as the internal control for normalization of the results.
and CYP11A1 expression level. But the much lower P4 level or P4 absence also suppressed folliculogenesis and ovulation [71, 72]. Concurrently, expression levels of cell proliferation related genes BCL-2 and CCND1 genes were significantly elevated in the GCs, whereas the expression of CASP3 gene was notably decreased. Furthermore, the cell proliferation ratio of the GCs was remarkably enhanced as compared to the negative control; conversely, cell apoptosis rate was sharply decreased as GABRA1 expression was silenced. Although the exact roles and regulatory mechanism of GABRA1 and the GABAR in ovarian follicular function remain unclear in chicken, a study reported the inhibitory effect of xanthohumol on evoked glutamate release was antagonized by suppressing the GABAR/Ca\textsuperscript{2+}-calmodulin/AC/cAMP/PKA cascade in rat hippocampus [73]. Moreover, the AC/cAMP/PKA pathway was involved in granulosa cell proliferation and differentiation of ovarian follicles in hen [74, 75]. Our result showed GABRA1 gene plays an inhibitory role in ovarian follicle development, follicle selection and differentiation by suppressing steroidogenesis, GC proliferation and stimulating cell apoptosis of SYF.

As aforementioned, it was found that the expression NDUFAB1 mRNA in the ovarian follicles of three developmental stages was down-regulated in the JB hens. Previous studies corroborated that NDUFAB1 is linked directly to mitochondrial ATP production, indirectly to potential reactive oxygen species generation [76], and reduced mitochondrial ATP production directly resulted in increased cell apoptosis [77]. Moreover, down-regulation of mitochondrial ACP expression in HEK293T cells has brought about a slowing of cell growth rate or a high level of cell death [78]. It was consistent with our results that the GC proliferation ratio was notably decreased; moreover, cell apoptosis rate was markedly increased when NDUFAB1 expression was silenced. Simultaneously, it was accompanied by the significant downregulation of STAR, CYP11A1, CCND1, and BCL-2 expression, and remarkably upregulated Caspase-3 expression. It confirmed that NDUFAB1 has a positive regulation in ovary follicular growth and differentiation at the developmental stage of follicle selection by enhancing steroidogenesis, GC proliferation and decreasing cell apoptosis. Furthermore, this study has shown the expression levels of NDUFAB1 mRNA across the three developmental stages of GWF, SYF and LYF in ovary development keeps lower in JB hens than in LB layers, but the higher expression of GABRA1 transcripts. Accordingly, it was concluded both NDUFAB1 and GABRA1 genes were strongly associated with egg-laying production of hens. Additionally, in this study three crucial signaling pathways including PPAR signaling pathway, cAMP signaling pathway, and neuroactive ligand-receptor interaction were screened out. Among them, the cAMP signaling pathway was not only involved in the neuroactive ligand-receptor interaction such as the GABAR/Ca\textsuperscript{2+}-calmodulin/AC/cAMP/PKA cascade of the GABAergic synapse pathway [54, 73], it also provokes a variety of processes
required for ovarian follicle growth, selection, differentiation, and maturation [74, 79]. As is well known, FSH is essential for reproductive system including steroidogenesis, folliculogenesis, and follicular maturation by interaction with FSH receptor (FSHR) in an endocrine dependent manner [80, 81]. FSHR, a member of the superfamily of G-protein-coupled receptors, is exclusively expressed on granulosa cells of ovarian follicles and mediates FSH signal transduction through cAMP signaling pathway [2, 28, 82]. The PPAR signaling pathway was previously reported to affect ovarian follicle development and normal ovarian function by being indirectly involved in oocyte maturation and ovulation via regulation of steroid hormone synthesis in granulosa cell [74]. Accordingly, the key candidate genes such as NDUFAB1, MC2R, VIPR2, GHRHR-LR, MC2R and SSTR2 that were identified currently may be implicated in granulosa cell proliferation and apoptosis, oocyte meiosis and maturation, follicular differentiation and atresia, and secretion of gonadotrophin-release hormone via crosstalk or intracellular interactions with the PPAR pathway [61, 79, 83–86].

**Conclusions**

Collectively, the transcriptome comparative analysis of ovarian follicles at the GWF, SYF and LYF developmental stages reveals the key genes and signaling pathways involved in ovarian follicular follicle growth (including follicle selection), differentiation, and maturation, which has provided molecular evidences for new insight into the regulatory mechanism underlying ovarian follicle development associated with egg production in chicken.

**Methods**

**Chicken raising management and trait measurement**

After hatching, LB and JB hens were raised in layered batteries under the same rearing conditions, including free access to water and feed in accordance with the recommendations for nutrient levels of in Lohmann breeds.
Approaching 16 weeks of age, hens were reared in individual cages under constantly maintained conditions. All of the layers were exposed to a 16 L: 8D photoperiod, with lights on at 5:00 AM. Age at first egg was recorded after the start of laying and egg production was observed daily, with egg weights determined on 1 day per week. Following feed and water restrictions at 21 and 66 weeks of age, BW was recorded and the individual laying performance calculated. Egg-laying traits examined in this study included hen-housed egg production (egg-laying number) at 21, 30, 43, 57, and 66 weeks of age. Average egg production rates, average egg weight and body weight of LB and JB hens at the ages were calculated and compared.

Samples preparation and cell culture
In this study, ten layers of every JB and LB breeds were obtained and euthanized at 21 weeks of age, and the GWF, SYF and LWF follicles were harvested. After the surrounding vascular and connective tissues of the follicles were removed with fine forceps and a scalpel [88], follicles were immediately snapped frozen in liquid nitrogen and preserved at −80 °C for RNA extraction. All experiments and methods were performed in accordance with the ARRIVE guidelines.

The GCs from the SYFs were isolated and cultured in Medium199 (M199; Gibco, Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum (Gibco) at 37 °C with 5% CO₂ in humidified chambers following our published method [8, 89]. The cultured GCs used in this experiment have been purified and quantified in our laboratory. The specificity of the GCs has been identified by the H & E staining procedure and fluorescence staining analysis [25, 90].

Total RNA was isolated from follicles of each hen using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the recommended manufacturer’s protocol, and concentration, quality, and integrity were determined by a Thermo NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). Three micrograms of RNA were used as input material for RNA sample preparations. The other parts of the samples were stored at −80 °C for validation experiments.

RNA-Seq analysis
The 18 cDNA libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the method published by our group [23]. Among them, nine cDNA libraries corresponding to the GWF (named A11, A12, A13), SYF (named A21, A22, A23) and LYF (named A31, A32, A33) samples from LB hens and nine cDNA libraries corresponding to the GWF (named B11, B12, B13), SYF (named B21, B22, B23) and LYF (named B31, B32, B33) samples from JB layers were constructed, respectively. Effective concentration of libraries were greater than 2 nM and libraries were
qualified, sequenced, and paired-end sequencing with the 150 bp sequencing read length was performed. The cDNA libraries were sequenced on a HiSeq platform (Illumina, Inc., San Diego, CA, USA) by Shanghai Personal Biotechnology Cp. Ltd. Follow-up analyses were based on clean data with high quality. All of the clean reads were aligned with the reference genome (ftp://ftp.ensembl.org/pub/release-86/fasta/gallusgallus/dna/Gallusgallus.Gallusgallus-5.0.dna.toplevel.fa.gz) by using sequence alignment program HISAT 2.1.0 [91, 92].

Identification of differentially expressed genes

In accordance with our earlier reported method [23], the HTSeq (ver. 0.6.1) software was utilized to count the reads mapped to each gene. The transcription level was normalized according to its FPKM (Fragments per Kilobase of transcript per Million mapped reads) values using the Cufflinks package (v2.1.1). And based upon the normalized FPKM + 1 value, expression pattern assessment for differentially expressed genes (DEGs) between case group and control group was fulfilled by using the Multi-Experiment Viewer (version 4.9.0) software (https://sourceforge.net/projects/mrv-tm4/files/latest/download). The resulting p-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate and an absolute value of the $|\log_2\text{FoldChange}|$ ($\log_2\text{FC}$) was served as the threshold for judging significance of the gene expression. Candidate genes with an adjusted $p$-value (padj) $< 0.05$ and $\log_2\text{FC} > 1$ were identified as differentially expressed. MA plots to visualize up-and down-regulated genes of each sample were generated by using the R package “ggplot2” (version 3.5.0), according to the values of padj and $\log_2\text{FC}$ [93].

GO annotation and KEGG enrichment analysis for DEGs

An online biological tool, Gene Ontology (GO, http://geneontology.org/), was utilized to annotate and analyze the molecular and functional characteristics of the DEGs [94]. The calculated $p$-value was Bonferroni corrected, taking the corrected $p$-value $< 0.05$ as a threshold.

---

**Fig. 9** Effects of GABRA1 silence on the GC proliferation and apoptosis. sh-GABRA1, GCs were transfected with GABRA1-specific shRNA; NC, scrambled shRNA; BC, no shRNA as a vehicle. A, B GABRA1 silence on the GC proliferation. C, D GABRA1 silence on cell apoptosis.
for GO annotation. Another online biological tool, Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/), provided the comprehensive database resources for the KEGG pathway enrichment of the DEGs. In this step, four databases were utilized to reveal high-level functions and biological systems of the DEGs, including Reactome (http://www.reactome.org), KEGG pathway (http://www.genome.jp/kegg/). Results with \( P < 0.05 \) were considered significantly enriched by DEG.

Data validation by quantitative real-time RT-PCR
To verify the accuracy and repeatability of the RNA-Seq results of DEGs, transcription levels of 24 representative genes in the ovarian follicles were estimated by using quantitative real-time reverse transcriptase PCR (RT-qPCR) as described previously [8]. The primers utilized for amplification of the candidate genes including VIPR2, GABRA1, PERP1, ZP1, and WISP12, et al., were listed in Table 5. Using the 2^(-ΔΔCt) method, mRNA expression results were normalized against 18S rRNA as internal control. To quantify mRNA expression levels by RT-qPCR analysis, four amplified products in independent reactions per bird were utilized. All the experiments were carried out in triplicate using different batches of sampled follicles.

Small hairpin RNA (shRNA) transfection
Three small interfering (siRNA) sequences targeting NDUFAB1 or GABRA1 gene were designed using an InvivoGen siRNA Wizard v3.1 and the most effective siRNA was screened out as we previously reported [8, 89]. After lentiviral expression vector pLVX-shRNA2-NDUFAB1 or -GABRA1 carrying the specific siRNA was constructed, GCs were then transfected with the NDUFAB1 shRNA or GABRA1 shRNA lentivirus in 24-well plates (2 × 10^5 cells/well), respectively; and incubated at 37°C with 5% CO2. After 24 h of culture, the GCs were collected for EdU cell proliferation and cell apoptosis assay, and lysed for Western blotting and RT-qPCR analysis. The sequence information of NDUFAB1 shRNA, GABRA1 shRNA, shRNA negative control and the frame of lentiviral vectors was shown in Table S2. The most effective siRNA sequences were listed as below: NDUFAB1 siRNA 5′-CCA CAA GAG AUA GUA GAU UTT-3′;

Table 5 Primer pairs designed for quantitative real-time PCR analysis

| Gene      | Forward primer (5′ - 3′) | Reverse primer (5′ - 3′) | Accession No. | Size |
|-----------|--------------------------|--------------------------|---------------|------|
| VIPR2     | ATA ATG ACT ATG AGG ACG AT | TGGATGATGTCCAGAGTA       | NM_001014970  | 154bp|
| GABRA1    | TGTGTTTCTTGCCCCTCATC    | ATCCTTCACCTTCCTTGCC    | NM_204318     | 109bp|
| PERP1     | AGACCTTGGCCTATGTGC    | GAAGTGAACAAGAGGTGTAT    | NM_001277703  | 206bp|
| ZP1       | CCTCACCATGTGGTCCAGGC | TGGCGTCAGGTTAGTAGGG      | NM_204683     | 222bp|
| WISP1     | CCAGGATTTCCAAGACAC     | GACAGCCAGCCTCTTTT      | NM_001024579  | 122bp|
| MC2R      | TCCTCTACCTTCTTCCGTA   | ACTTGTTGAGTGTAGGCC       | NM_001031515  | 245bp|
| STAR    | AGTGGCAGATCGTGAACAC     | AGTGGGACTGCTAGTAGAT       | NM_001079742  | 249bp|
| NDUFAB1  | AGGAGGATGGTGGTCTTTTG   | TGACATCAGGGCCATCC         | XM_004945263  | 157bp|
| BCL2L12  | TAAAGAACACACAGAATCT    | TGAGAGCAGCTGGCAAG        | XM_004060909  | 231bp|
| LOCA24011 | TGGAGGATGCTGGTGTA | AATGGTGATGGTCTGTAGTG     | XM_001232693  | 130bp|
| ADRB2    | GAGAGCAGTACAACAGGAGG  | AATCATCCCCAACGGACCAC     | XM_004683024  | 255bp|
| PALL     | GCAGTAGATGAAAGCGATG    | GCCATACCCAACTCCAC        | NM_001165912  | 172bp|
| HSD17B1  | CAGGCGCAGGCCACATCTCA   | CGCAGTCCACACGCTGTAT       | NM_204837     | 206bp|
| NCAM2   | CGGCTACAACAAAGAAATAGGAA | ATGATGGAAACCTGCGAGGAT | XM_425540     | 124bp|
| CYP2006  | TTACTACAACCGCCATCT   | AAGGACTTTCCTCCTCAT      | NM_001195557  | 297bp|
| CRH     | CTGAGACCTGATTTCCACCCTGT | CGGCTTACCTGCAGTGAGTA  | NM_001123031  | 116bp|
| GHRHR-LR | TGCCATCTTCTCCAGTCA    | GACCAAGTAAAGCATCACAG      | XM_004664932  | 167bp|
| ID4      | ACAAGGGGTCGCTGAGAGG    | TACGCGGCTGGTGTTAGGGG     | NM_204282     | 179bp|
| SSTR2   | CCAACTCGAGGGAACAAGAC  | CAAAGGGAGCAGTCAACA       | NM_001030345  | 164bp|
| CDKN1A   | GACAGCAGGAAGGACTGAG    | AGACGGGAGAGCAGCACA       | NM_204396     | 129bp|
| STAR    | GCCTCGAGCAGCAGAAGT    | TCCCTACTTGGATCCCTGAT       | NM_204686     | 196bp|
| CYP11A1  | GCTTGGTCCTGAGCTGTTG   | TGGGGTCCTGCTGCTGTTG       | NM_001001756  | 277bp|
| CCN1    | GGCAGCAAGATGCGAAGGAGG  | TGGAGTGGTGGTGAATTG     | NM_205381     | 188bp|
| BCL-2    | ATGACCCGAGCTCTGACAGC   | CCAAGTAAAGGAGGAGATG      | NM_205339     | 182bp|
| CASP3   | AAGAATCTCCACCCAGATACCG | GCTTACGCAACACCAACAAAAA  | NM_204725     | 204bp|
| 18S rRNA | ATGGAGGGCCAGAATCTGTTG  | CCAGCTCGATCCCAAGATCC     | AF173612      | 109bp|
siRNA: 5′- UUCUCCGAAACGUACGUTT-3′. Scrambled siRNA was used as the negative control siRNA: 5′- UUUCCGAAUCGUACGUTT-3′, which was synthesized by GenePharma (Shanghai, China).

Western blotting
Western blot analysis for STAR, CYP11A1, CCND1, BCL-2, and Caspase-3 was performed using total cellular extracts according to our previously described [8, 89]. The affinity-purified antibodies for STAR and the other antibodies were used (Supplementary Table S3). Briefly, equal amounts of protein were separated by 10% (w/v) SDS-polyacrylamide gel under reducing conditions and electro-transferred to Protran nitrocellulose membranes (Whatman, Dassel, Germany). After electrophoresis of the protein samples in a mini gel apparatus, a pre-stained protein molecular weight marker was loaded to locate/monitor the target proteins in the electrophoresis (SDS-PAGE). At the approximated protein size position, the electro-transferred to Protran nitrocellulose membranes. The horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody was incubated for 2h at room temperature. Blots were subsequently performed with ECL western blotting agent (Rockford, IL, USA) for 5 min and exposed to X-ray film for 1–5 min. The signals were detected using the ECL Plus Western blotting detection system according to the manufacturer’s instructions. Anti-β-actin (dilution 1:500, Boster, Wuhan, China) antibody acted as a loading control. As a result, the molecular size of the ladders was not observed in the original blots.

EdU cell proliferation assay
Following the shRNA transfection of the GCs with NDUFA1 or GABRA1 silence, a variation in cell proliferation was determined by EdU (5′-Ethynyl-2′- deoxyuridine) incorporation assay using the Cell-Light EdU imaging kit (RiboBio, Guangzhou, China) according to the manufacturer’s protocol. Each experiment was performed in triplicate and repeated five times. The number of EdU-positive cells was expressed as a percentage and calculated relative to the total number of cells (positive cells/total cells in one field) as previously described [8]. The ratio of positive cells was calculated from the average of the five group values.

Cell apoptosis assay
The apoptosis of GCs was determined by flow cytometry (BD Biosciences, USA) using the Annexin V- Allophycocyanin(APC)/7-amino-actinomycin D (AAD) Apoptosis Detection Kit (KeyGEN, Nanjing, China), in accordance with the manufacturer’s protocols. The apoptosis rate of GCs was estimated, i.e., total number of apoptotic cells comprises the number of cells in the right upper quadrant (Q1-UR) and number of cells in the right lower quadrant (Q1-LR), and analyzed using FlowJo v7.6 software (Stanford University, Stanford, CA, USA). Each experiment was carried out for at least three times.

Statistical analysis
Statistical analysis was fulfilled using the SPSS12.0 software package [95]. Data were analyzed by executing a Student’s t-test for comparisons between the RNA-Sequencing and RT-qPCR determination after confirmation of normal distributions for non-parametric analysis. Values were presented as mean ± SEM and bars with superscript symbols that indicate the significant difference compared with control groups at *** p < 0.001, ** p < 0.01, * p < 0.05.

Abbreviations
NDUFA1: NADH: ubiquinone oxidoreductase subunit AB; GABRA1: Gamma-aminobutyric acid type A receptor alpha1 subunit; STAR: Steroidogenic-related enzymes steroidogenic acute regulatory protein; HSD17B1: Hydroxysteroid (17βeta) dehydrogenase 1; P450scc/CYP11A1: Cytochrome P450 side-chain cleavage; P4: Progesterone; E2: Estradiol; AMH: Anti-müllerian hormone; CCND1: Cyclin D1; CASP3: Caspase-3; Bcl-2: B-cell lymphoma-2; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; GWF: Growing white follicle; SYF: Small yellow follicle; LYF: Large yellow follicle; DEG: Differentially expressed gene; GC: Granulosa cell; JB: Jilin Black chicken; LB: Lohmann Brown chicken; shRNA: Small interfering RNA; siRNA: Small hairpin RNA; EdU: 5′-Ethynyl-2′- deoxyuridine; APC: Annexin V- Allophycocyanin; AAD: 7-amino-actinomycin D; AF: Age at first egg; BW: Body weight; EW: Egg weight; HH: Hen-housed egg production; EN: Egg numbers per hen; cAMP: Adenosine-3′,5′-cyclic monophosphate; CAM: Cell adhesion molecule; AMPK: AMP-activated protein kinase; FSH: Follicle stimulating hormone (FSH); FSHR: FSH receptor; PACAP: Adenylyl cyclase-activating polypeptide; VIP: Vasoactive intestinal peptide; PAC1: PACAP type 1 receptor; VIPR: VIP receptor; TC: Theca cell; ADRB2: β2 adrenergic receptor; PKA: Protein kinase A; PKC: Protein kinase C; PLC: Phospholipase C; CREB: cAMP response element-binding protein; PCOS: Polycystic ovary syndrome; ACP: Acryl carrier protein.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-08213-w.

Additional file 1: Supplementary Table S1. Results of the cleaned data of reads filtered and the reads mapped. Supplementary Table S2. Construction frame of lentiviral vectors against NDUFA1 and GABRA1 genes. Supplementary Table S3. Antibodies used for western blot analysis. Supplementary Figure S1. The oxidative phosphorylation pathway (gga: 00190) and its components. Supplementary Figure S2. The neuroactive ligand-receptor interaction pathway (gga: 04080) and its components. Supplementary Figure S3. The images show the original gels of western blot in the Fig. 6E and Fig. 6F. Supplementary Figure S4. The images show the original gels of western blot in the Fig. 8B and Fig. 8E.

Acknowledgements
We would like to thank the help of sequencing analysis from Personal Biotechnology Co., Ltd. Shanghai, China.

Authors’ contributions
XS and XXC contributed equally to this manuscript. XS and XXC mainly performed the experiments; JHZ, CM, CCY and SL analyzed the data, and helped...
perform some experiments; RFX conceived, designed the experiments, and wrote the manuscript; NQ designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

Funding
RFX is funded by grants from the National Natural Science Foundation of China (Grant No.321272714, No.31672407), the Project of Science and Technology Development Plan of Jilin Province (No. 20170101019JC), and the China Agriculture Research System of MOF and MARA (No.CARS-45-203). NQ is funded by grants from the National Natural Science Foundation of China (Grant No.31902145), and the Project of Education Development Plan of Jilin Province (No. JJKH2018064GKJ).

Availability of data and materials
The relevant datasets have been published by our group and are available at PRJNA669967 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA669967?reviewer=1qgd76c8xv7u2cd3r3un4vm) and PRJNA670553 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA670553?reviewer=3fu2up6df8af1h9sue09nypc) in NCBI [23].

Declarations
Ethics approval and consent to participate
This study is in accordance with ARRIVE guidelines. All procedures executed in chickens were approved by the Animal Ethics Committee of Jilin Agricultural University (Changchun, China). Ethical approval for animal survival was given by the Animal Ethics Committee of Jilin Agricultural University (Changchun, China). All birds were sacrificed prior to the removal of organs based upon the IACUC Guidelines for the euthanasia of experimental animals (Permission No. GR (J) 19-030).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Joint Laboratory of Modern Agricultural Technology International Cooperation, Ministry of Education, Jilin Agricultural University, Changchun 130118, China. 2 Department of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China.

Received: 4 July 2021 Accepted: 26 November 2021

Published online: 15 December 2021

References
1. Johnson PA. Follicle selection in the avian ovary. Reprod Domest Anim. 2022;47(Suppl 4):283–7.
2. Johnson AL. Ovarian follicle selection and granulosa cell differentiation. Poult Sci. 2015;94(4):781–9.
3. Brady K, Porter TE, Liu HC, Long JA. Characterization of the hypothalamic-ovarian-pituitary-gonadal axis in low and high egg producing Turkey hens. Poult Sci. 2020;99(2):1163–73.
4. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod. 1991;44(2):305–14.
5. Klein-Hessling H. Managing breeder hens for more eggs. World Poultry. 2007;23(5):16–7.
6. Woods DC, Johnson AL. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol Reprod. 2005;23(3):643–50.
7. Shen M, Li T, Chen F, Wu P, Wang Y, Chen L, et al. Transcriptional analysis of circRNAs and mRNAs reveals a complex regulatory network that participate in follicular development in chickens. Front Genet. 2020;11:503.
8. Xu R, Qin N, Xu X, Sun X, Chen X, Zhao J. Inhibitory effect of SLIT2 on granulosa cell proliferation mediated by the CDC42-PAKs-ERK1/2 MAPK pathway in the prehierarchical follicles of the chicken ovary. Sci Rep. 2018;8(1):9168.
9. Johnson AL, Solovey EV, Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biol Reprod. 2002;67(4):1313–20.
10. Sechman A, Pawlowska K, Hrabia A. Effect of 3,3′,5-triiodothyronine and 3,3′,5-diiodothyronine on progesterone production, cAMP expression, and mRNA expression of STAR, CYP1A1, and HSD3B genes in granulosa layer of chicken preovulatory follicles. Domest Anim Endocrinol. 2011;41(3):137–49.
11. Ocón-Grove OM, Poole DH, Johnson AL. Bone morphogenetic protein 6 promotes FSH receptor and anti-Müllerian hormone mRNA expression in granulosa cells from hen prehierarchical follicles. Reproduction. 2012;143(6):825–33.
12. Huang J, Cui H, Peng X, Fang J, Zuo Z, Deng J, et al. The association between splenocyte apoptosis and alterations of Baz, Bcl-2 and caspase-3 mRNA expression, and oxidative stress induced by dietary nickel chloride in broilers. Int J Environ Res Public Health. 2013;10(12):7310–26.
13. Heinosalo T, Saarinen N, Poutanen M. Role of hydroxycholesterol (17Beta) dehydrogenase type 1 in reproductive tissues and hormone-dependent diseases. Mol Cell Endocrinol. 2019;489:9–31.
14. Yang KT, Lin CY, Huang HL, Loui JS, Chen CY, Wu CP, et al. Expressed transcripts associated with high rates of egg production in chicken ovarian follicles. Mol Cell Probes. 2008;22(1):47–54.
15. Diaz FJ, Anthony K, Halfhill AN. Early avian follicular development is characterized by changes in transcripts involved in steroidogenesis, paracrine signaling and transcription. Mol Reprod Dev. 2011;78(3):212–23.
16. Wang Y, Chen Q, Liu Z, Guo X, Du Y, Yuan Z, et al. Transcriptome analysis on single small yellow follicles reveals that Wnt5a is involved in chicken follicle selection. Front Endocrinol (Lausanne). 2017;8:317.
17. Chen Q, Wang Y, Liu Z, Guo X, Sun Y, Kang L, et al. Transcriptomic and proteomic analyses of ovarian follicles reveal the role of VLDLR in chicken follicle selection. BMC Genomics. 2020;21(1):486.
18. Zhong C, Liu Z, Qiao X, Kang L, Sun Y, Jiang Y. Integrated transcriptomic analysis on small yellow follicles reveals that Sosondowah Ankyrin repeat domain family member a inhibits chicken follicle selection. Anim Biosci. 2021;34(8):1290–302.
19. Wang Y, Shi H, Zhang G, Wu P, Chen L, Shen M, et al. Transcriptome analysis of long noncoding RNAs and mRNAs in granulosa cells of Jinghai yellow chickens illuminated with red light. Front Genet. 2021;12:563623.
20. Luan X, Liu D, Cao Z, Luo L, Liu M, Gao M, et al. Transcriptome profiling identifies differentially expressed genes in Huoyan goose ovaries between the laying period and ceased period. PLoS One. 2014;9(11):e115321.
21. Ren J, Sun C, Chen L, Hu J, Huang X, Liu X, et al. Exploring differentially expressed key genes related to development of follicle by RNA-seq in Peking ducks (Anas Platyrhynchos). PLoS One. 2019;14(6):e0209061.
22. Brady K, Liu HC, Hicks JA, Long JA, Porter TE. Transcriptome analysis during follicle development in Turkey hens with low and high egg production. Front Genet. 2021;12:619196.
23. Chen X, Sun X, Chimbaka IM, Qin N, Xu X, Liwansiso S, et al. Transcriptome analysis of ovarian follicles reveals potential pivotal genes associated with increased and decreased rates of chicken egg production. Front Genet. 2021;12:622751.
24. Lohmann Tierzucht GmbH. Management guide for laying hens: Lohmann Brown-classic. Cuxhaven: Lohmann Tierzucht GmbH; 2005.
25. Qin N, Fan XC, Xu XX, Tsaiy LI, Li SJ, Zhang YY, et al. Cooperative effects of FOXO2 with the members of TGF-β superfamily on FSH receptor mRNA expression and granulosa cell proliferation from hen prehierarchical follicles. PLoS One. 2015;10(10):e1014062.
26. Gilchrist RB, Ritter LJ, Myllymäki S, Kaivo-Oja N, Dragovic RA, Hickey TE, et al. Molecular basis of oocyte-paracrine signaling that promotes granulosa cell proliferation. J Cell Sci. 2006;119(Pt 18):3811–21.
27. Woodruff TK, Shea LD. The role of the extracellular matrix in ovarian follicle development. Reprod Sci. 2007;14(Suppl 6):1–10.
28. Johnson AL, Woods DC. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. Gen Comp Endocrinol. 2009;163(1–2):12–7.
29. Son WY, Das M, Shalom-Paz E, Holzer H. Mechanisms of follicle selec-
tion and development. Minerva Ginecol. 2011;63(2):89–102.
30. Conconi MT, Spinazzi R, Nussdorfer GG. Endogenous ligands of PACAP/
VIP receptors in the autoinhibitory regulation of the adrenal gland. Int Rev Cytol. 2006;249:1–51.
31. Dickson L, Finlayson K, VPAC and PAC receptors: from ligands to func-
tion. Pharmacol Ther. 2009;121(3):294–316.
32. Heindel JJ, Snedeen J, Powell CJ, Davis B, Culler MD. A novel hypo-
thalamic peptide, pituitary adenylate cyclase-activating peptide, regulates the function of rat granulosa cells in vitro. Biol Reprod. 1996;54(3):523–50.
33. Apa R, Lanzone A, Miceli F, Vaccari S, Macchione E, Stefanini M, et al. Pitu-
itary adenylate cyclase-activating polypeptide modulates plasmogen activator expression in rat granulosa cell. Biol Reprod. 2002;66(3):830–5.
34. Vaccari S, Latini S, Barberi M, Teti A, Stefanini M, Canipari R. Characteriza-
tion and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. J Endocrinol. 2006;191(1):287–99.
35. Zhou R, Tsang AH, Lau SW, Ge W. Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors in the zebrafish ovary: evidence for potentially dual roles of PACAP in controlling final oocyte maturation. Biol Reprod. 2011;85(3):615–25.
36. Lara HE, Dissen GA, Leyton V, Paredes A, Fuensalida H, Fiedler JL, et al. An
increased intracellular synthesis of nerve growth factor and its low affinity
receptor is a principal component of steroid-induced polycystic ovary in the rat. Endocrinology. 2000;141(3):1059–72.
37. Parra C, Fiedler JL, Luna SL, Greiner M, Padmanabhan V, Lara HE. Participa-
tion of vasoactive intestinal polypeptide in ovarian steroids production during the rat estrous cycle and in the development of estradiol valerate-induced polycystic ovary. Reproduction. 2007;133(1):147–54.
38. Mayerhofer A, Dissen GA, Costa ME, Ojeda SR. A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinol-
yogy. 1997;138(8):3210–9.
39. Merz C, Saller S, Kunz L, Xu J, Yeoman RR, Ting AY, et al. Expression of the
beta-2 adrenergic receptor (ADRB2-2) in human and monkey ovarian fol-
licles: a marker of growing follicles? J Ovarian Res. 2015;8:8.
40. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, et al. Chronic
stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006;12(8):939–44.
41. Kang Y, Nagaraja AS, Armaiz-Pena GN, Domiak PL, Hu W, Rupaimole R, et al. Adrenaline stimulation of DUSP1 impairstherapy response in ovarian cancer. Clin Cancer Res. 2016;22(7):1713–24.
42. Hatefi A, Zare Shahneh A, Ansari Pirsaraie Z, Alizadeh AM, Atashnak MP, et al. The stimulation and inhibition of beta-2 adrenergic
receptor on the inflammatory responses of ovary and immune system in the aged laying hens. BMC Vet Res. 2021;7(1):195.
43. Barras A, Leyton V, Ojeda SR, Lara HE. Ovarian steroid response to gonadotropins and beta-adrenergic stimulation is enhanced in polycystic ovary syndrome: role of sympathetic innervation. Endocrinology. 1993;133(6):2696–703.
44. Wang Y, Fu X, Xu J, Wang Q, Kuang H. Systems pharmacology to investig-
te the interaction of berberine and other drugs in treating polycystic ovary syndrome. Sci Rep. 2016;6:28089.
45. Labrie F, Luo-The V, Lin SX, Labrie C, Simard J, Breton R, et al. The key
components of the GnRH/FSH/LH axis: insights from a systems approach. J Endocrinol. 2014;222(1):23–32.
46. Delvoux B, D’Hooghe T, Kyama C, Koskimies P, Hermans RJ, Duns
elman GA, et al. Inhibition of type 1 17β-hydroxysteroid dehydrogenase in the synthesis of 17β-estradiol in endometriosis lesions. J Clin Endocrinol Metab. 2014;99(1):276–84.
47. Shen JM, Chen X, Liang H, Wang W, Wang D, Feng Z, et al. The expression and function of a novel estrogen receptor in human ovarian cancer. Mol Endocrinol. 2014;28(8):1653–67.
48. Hilborn E, Stål O, Jansson A. Estrogen and androgen-converting enzymes
in the ovary. Mol Cell Endocrinol. 2006;248(1–2):141–8.
49. Barberi M, Muicicca B, Morelli MB, Stefanini M, Geconni S, Canipari R. Expression
localisation and functional activity of pituitary adenylate cyclase-activatingpolypeptide, vasoactive intestinal peptide and their receptors in mouse ovary. Reproduction. 2007;134(2):281–92.
50. Zhou M, Lei M, Yao Q, Nie Q, Zeng H, Xia M, et al. Polymorphisms of
vasoactive intestinal peptide receptor-1 gene and their genetic effects on broodiness in chickens. Poul Sci. 2008;87(5):893–903.
51. Zhou M, Du Y, Nie Q, Liang Y, Luo C, Zeng H, et al. Associations between polymorphisms in the chicken VIP gene, egg production and broody
traits. Br Poul Sci. 2010;51(2):195–203.
52. Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SM, et al. Atomic
force microscopy reveals the stoichiometry and subunit arrangement of the alpha1beta3delta GABA(A) receptor. Mol Pharmacol. 2008;73(3):960–77.
53. Huang SH, Reddy DS. Genetic and molecular regulation of extrasymp-
tatic GABA-A receptors in the brain: therapeutic insights for epilepsy. J Pharmacol Exp Ther. 2018;364(2):180–97.
54. Olsen RW, Sieghart W. GABA: a receptor subtypes provide diversity of function and pharmacology. Neuropharmacology. 2009;56(1):141–8.
55. Sigel E, Steinmann ME. Structure, function, and modulation of GABA(A)
receptors. J Biol Chem. 2012;287(4):2604–31.
56. Sexton CA, Penzinger R, Mortensen M, Bright DP, Smart TG. Structural
determinants and regulation of spontaneous activity in GABA(A) receptors. Nat Commun. 2021;12(1):5457.
57. Bixo M, Johansson M, Timby E, Michalski L, Backström T. Effects of GABA
active steroids in the female brain with a focus on the premenstrual dys-
phoric disorder. J Neuroendocrinol. 2018;30(2). https://doi.org/10.1111/jne.12553.
58. Tuem KB, Atey TM. Neuroactive steroids: receptor interactions and
responses. Front Neuro. 2017;8:442.
59. Masud AJ, Kastaniotis AJ, Rahman MT, Auto JK, Hiltunen JK. Mitochon-
drial acyl carrier protein (ACP) at the interface of metabolic state
sensing and mitochondrial function. Biochim Biophys Acta Mol Cell Res. 2019;1866(12):118560.
60. Byers DM, Gong H. Acyl carrier protein: structure-function relationships
in a conserved multifunctional protein family. Biochem Cell Biol. 2007;85(6):649–62.
61. Chai DI, Vogel HJ. Current understanding of fatty acid biosynthesis and the acyl carrier protein. Biochem J. 2010;430(1):11–19.
62. Hiltunen JK, Chen Z, Haapalainen AM, Wierenga RK, Kastaniotis AJ. Mitochondrial fatty acid synthesis - an adopted set of enzymes making
a pathway of major importance for the cellular metabolism. Prog Lipid Res. 2010;49(1):27–49.
63. Johnson AL, Bridgman JT. Regulation of steroidogenic acute regulatory
protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology. 2001;142(7):3116–24.
64. Tilly JL, Kowalski Kl, Johnson AL. Cytochrome P450 side-chain cleavage
(P450ccc) in the hen ovary. II. P450ccc messenger RNA, immunoreactive protein, and enzyme activity in developing granulosa cells. Biol Reprod. 1991;45(6):967–74.
65. Robinson FE, Etches RJ. Ovarian steroidogenesis during follicular
maturation in the domestic fowl (Gallus domesticus). Biol Reprod. 1986;35(5):1096–105.
66. Gutierrez CG, Campbell BK, Webb R. Development of a long-term bovine
gland. Int Rev Cytol. 2006;248(1–2):141–8.
67. Zalán J, Gómez-González MA, Haukkamaa M, Lähteenmäki P. Inhibition
of folliculogenesis and ovulation by the antiprogesterone RU 486. Fertil Steril. 1988;50(4):961–3.
68. Heinkheim O, Gordon K, Williams RF, Hodgen GD. Inhibition of ovulation by agonist (agonists vs antagonists): preliminary
evidence for different sites and mechanisms of actions. Contraception. 1996;53(1):55–64.

73. Chang Y, Lin TY, Lu CW, Huang SK, Wang YC, Wang SJ. Xanthohumol-induced presynaptic reduction of glutamate release in the rat hippocampus. Food Funct. 2016;7(1):212–26.

74. Dupont J, Reverchon M, Cloix L, Froment P, Ramé C. Involvement of adipo-kinases, AMPK, PI3K and the PPAR signaling pathways in ovarian follicle development and cancer. Int J Dev Biol. 2012;56(10–12):959–67.

75. Nemer A, Azab AN, Rimon G, Lamprecht S, Ben-Menahem D. Different roles of CAMK/PI3K and PI3K signaling in regulating progesterone and PGE2 levels in immortalized rat granulosa cell cultures. Gen Comp Endocrinol. 2018;269:88–95.

76. Pecorelli A, Leonii G, Cervellati F, Canali R, Signorini C, Leoncini S, et al. Genes related to mitochondrial functions, protein degradation, and chomatin folding are differentially expressed in lymphomonocytes of Rett syndrome patients. Mediat Inflammam. 2013;2013:37629.

77. Yu Z, Poppe JL, Wang X. Mitochondrial mechanisms of neuroglobin’s neuroprotection. Oxidative Med Cell Longev. 2013;2013:756989.

78. Feng D, Witkowski A, Smith S. Down-regulation of mitochondrial acyl carrier protein in mammalian cells compromises protein lipolyti- tion and respiratory complex I and results in cell death. J Biol Chem. 2009;284(17):11436–45.

79. Richards JS, Ascoli M. Endocrine, paracrine, and autocrine signaling pathways that regulate ovulation. Trends Endocrinol Metab. 2018;29(5):313–25.

80. Choi JH, Choi KC, Auerpersp N, Leung PC. Gonadotropins upregulate the epidermal growth factor receptor through activation of mitogen-activated protein kinases and phosphatidylinositol-3-kinase in human ovarian surface epithelial cells. Endocr Relat Cancer. 2005;12(2):407–21.

81. Johnson AL. Reproduction in the female. In: Whittow GC, editor. Sturkie’s avian physiology (fifth edition). New York: Academic Press; 2015. p. 569–96.

82. Simoni M, Gromoll J, Nieszlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr Rev. 1997;18(6):739–73.

83. Amstredam A, Keren-Tal I, Aharoni D. Cross-talk between cAMP and p33-generated signals in induction of differentiation and apoptosis in steroidogenic granulosa cells. Steroids. 1996;61(4):252–6.

84. Nakanishi T. Endocrine disruption induced by organotin compounds; organotins function as a powerful agonist for nuclear receptors rather than an aromatase inhibitor. J Toxicol Sci. 2006;31(3):269–76.

85. Liu YX, Zhang Y, Li YY, Liu XM, Wang XX, Zhang CL, et al. Regulation of csal1 and csal3 in granulosa cell proliferation and steroidogenesis of hen ovarian prehierarchical development. Biol Reprod. 1990;42(5–6):747–54.

86. Hanlon C, Ramachandran R, Zuidhof MJ, Bédécarrats GY. Should I lay or should I grow: photoperiodic versus metabolic cues in chickens. Front Physiol. 2020;11:707.

87. Thiele HH. Management recommendations for rearing pullets for alterna-tive housing systems. Lohmann Inf. 2007;42(2):14–24.

88. Politis I, Wang L, Turner JD, Tsang BK. Changes in tissue-type plasminogen activator-like and plasminogen activator inhibitor activities in granulosa and theca layers during ovarian follicle development in the domestic hen. Biol Reprod. 1990;42(5–6):747–54.

89. Zhu H, Qin N, Xu X, Sun X, Chen X, Zhao J, et al. Synergistic inhibition of csgal1 and csgal3 in granulosa cell proliferation and steroidogenesis of hen ovarian prehierarchical development. Biol Reprod. 2019;101(5):986–1000.

90. Lyu Z, Qin N, Tyasi TL, Zhu H, Liu D, Yuan S, et al. The hippo/MST pathway member SAV1 plays a suppressive role in development of the prehierar-chical follicles in hen ovary. PLoS One. 2016;11(8):e0160896.

91. Perte M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016;11(9):1650–67.

92. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Bio-technol. 2019;37(8):907–15.

93. Wang L, Feng Z, Wang X, Wang X, Zhang X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinfor-matics. 2010;26(1):136–8.

94. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The gene ontology consor-tium. Nat Genet. 2000;25(1):25–9.

95. Lin JB, Chen X, Liu MD. SPSS 11.0 statistical analysis actual practice design (in Chinese). Beijing: Chinese Railroad Publishing House, China; 2002.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.