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RESEARCH ARTICLE

IGF2 Activates the PI3K-AKT Signaling Pathway to Promote Proliferation of Duck Ovarian Granulosa Cells

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ABSTRACT

To elucidate the specific role and mechanism of IGF2 (a strongly selected gene in egg-production traits identified in our previous genome resequencing of Chinese Jinding ducks) in duck ovaries, this study investigated the expression and distribution of IGF2, assessed its impact on the proliferation of duck ovarian granulosa cells (GC), and elucidated the underlying regulatory mechanisms. The expression of IGF2 mRNA in the ovary was significantly higher than in all other tissues ($P<0.05$), being 5.69 times that in the liver the tissue with the second-highest expression. Its protein was mainly located in GC, oocyte cytoplasm and vascular endothelium of duck follicles. *In vitro* experiments demonstrated that IGF2 enhanced the proliferation of duck GC. Further mechanistic analysis showed that IGF2 promoted the phosphorylation of PI3K and AKT, as well as the expression of downstream CCND3 gene, a cell cycle regulation gene. These findings suggested that IGF2 regulated duck GC proliferation through the PI3K/AKT/*CCND3* pathway, thereby affecting the laying traits of ducks. These results revealed a regulatory role of IGF2 in duck follicular development via the PI3K–AKT pathway and extended the existing understanding of IGF2 function in ducks.

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INTRODUCTION

In recent years, duck eggs have gained popularity due to their high nutritional value, leading to an increased demand. Egg production is a crucial economic trait for assessing duck reproductive performance (Zhou *et al*., 2024) and is governed by a complex interplay of multiple genes. Follicular development was precisely regulated by various signals from oocytes (e.g., BMP15, GDF9) and the neuroendocrine system (e.g., GnIH), which together coordinated the functional status of granulosa cells (Scanes, 2022; Yu *et al*., 2025). Several genes related to egg production have been identified using molecular-assisted methods, providing valuable references for the genetic improvement of laying ducks. IGF2 has been identified as one of the candidate genes associated with egg laying in chickens and geese (Zhao *et al.*, 2023; Tan *et al.*, 2024). Furthermore, in our previous work, performed genome resequencing of laying ducks (Jinding ducks) and traditional broiler ducks (Pekin ducks), have revealed strong selection signals and multiple mutation sites in the insulin-like growth factor 2 (*IGF2*) gene on chromosome 5. That suggested its potential involvement in egg-production traits, although the precise regulatory mechanisms remain unclear.

The insulin-like growth factor (IGF) family, comprising *IGF1* and *IGF2*, were multifunctional cellular regulators that mediated growth hormone stimulation and govern tissue growth and development (Yang *et al*., 2022). IGF have dynamic regulatory influence throughout poultry embryonic development (Wang *et al*., 2024). *IGF2* assumed a critical role during pregnancy, exhibiting high expression levels in mouse embryos and placenta (Lopez-Tello *et al*., 2023). Destruction of *IGF2* expression led to delayed fetal growth (Sélénou *et al*., 2022). In certain scleractinian fish species, IGFs, particularly *IGF2*, were highly expressed in the ovaries, regulating ovarian development (Irwin and Van Der Kraak, 2012). Notably, IGF2 was the sole IGF gene expressed in human granulosa cells (GCs), where it synergized with follicle-stimulating

hormone to enhance the activation of the insulin-like growth factor 1 receptor (IGF1R), thereby inducing GC proliferation and differentiation (Hensen *et al*., 2020; Hobeika *et al*., 2020; Zhao *et al*., 2024). Protein kinase B (AKT) activity was pivotal in this process (Baumgarten *et al*., 2015; Forbes *et al*., 2020). Avian reproduction was also regulated by the IGF family. IGF1 regulated granulosa cell apoptotic signaling and affected follicular atresia in aged chickens (Ru *et al*., 2024). Moreover, the overexpression of insulin-like growth factor binding proteins inhibited the proliferation and differentiation of GCs, and the associated lipid metabolism disorder and excessive autophagy might lead to GC degeneration (Zhang *et al*., 2024). In Muscovy ducks, *IGF2* mRNA expression in the ovary was found to correlate with egg production (Ye *et al*., 2017). *IGF2* could promote the proliferation of chicken ovarian GC, inhibited the apoptosis of GC, and promote the development of chicken follicles (Zhao *et al*., 2025). Furthermore, *IGF2* may play an important role in follicle selection. Both IGF1 and IGF2 can induce rapid phosphorylation of AKT signaling in chicken granulosa cells and regulate their proliferation and apoptosis through the PI3K–AKT pathway (Xin *et al*., 2022; Ru *et al*., 2024; Zhao *et al*., 2025). Previous studies on the regulatory mechanisms of IGF2 in ovarian function have primarily concentrated on mammals and chickens in poultry, with limited investigations conducted on ducks. The function and underlying mechanisms of *IGF2* within duck ovaries, particularly its impact on GC proliferation, follicle development, and the involved signaling pathways, remain elusive.

Consequently, we hypothesized that *IGF2* regulated GC proliferation and follicle development via the PI3K-AKT signaling pathway, ultimately influencing egg-laying performance in ducks. This research aimed to investigate the tissue-specific expression and distribution pattern of IGF2 in duck and reveal its role and regulatory pathway in regulating the proliferation of duck GCs.

MATERIALS AND METHODS

Animals: The Jinding ducks used in this study were obtained from the Germplasm Resource Center, Fujian Academy of Agricultural Sciences, Fujian Province, China. During the peak laying period, ten adult female ducks were selected and euthanized for issue collection. All animal procedures were conducted in accordance with the guidelines and regulations of the Ethics Committee for the Care and Use of Animals of the Institute of Animal Husbandry and Veterinary Research, Fujian Academy of Agricultural Sciences (Approval NO. 202402GJ012).

Protein expression: The coding sequence of *IGF2* (accession number: XM_027457601.2) was amplified using duck cDNA as a template and inserted between the EcoRV and BamHI restriction enzyme sites. The corresponding primer sequences were listed in Table 1. The *pET32–IGF2* vector was successfully constructed, and the expressed *pET32-IGF2* products were subsequently identified and purified.

Hematoxylin and eosin (HE) staining: Small yellow follicles were washed with PBS and fixed in 4% paraformaldehyde (Servicebio, Wuhan, China). Dehydration was performed using a graded ethanol series as follows: 75, 85, 90, and 95% ethanol for 4, 2, 2, and 1 h, respectively; absolute ethanol for 30 min (twice); and alcohol–xylene mixture for 5–10 min (twice). After dehydration, the tissues were embedded in paraffin and sectioned into 8-μm-thick slices. Sections were stained with hematoxylin (Servicebio,Wuhan, China) for 3–5 min, rinsed with tap water, dehydrated with 95% ethanol, stained with eosin (Servicebio, Wuhan, China) for 15 s, and further dehydrated through an ethanol gradient before microscopic observation.

Table 1: Primers for *IGF2* coding sequence

Primers name	Sequence(5′ to 3′)	Primers size (bp)	Expected amplicon sizes (bp)
IGF2-F	GACAAGGCCATGGCTGATATCG CGTACGGCACGGCGGAGA	40	207
IGF2-R	ACGGAGCTCGAATTCGGATCCC TACTCAGACTTGACGGACTTGG	44	

Note: Underlines on primer sequence represent EcoRV cleavage site (*IGF2*-F) and BamHI cleavage site (*IGF2*-R).

Immunohistochemistry: The tissue sections were subjected to deparaffinization, antigen retrieval, endogenous enzymes inactivation, and serum blocking. They were then incubated with the primary antibody (human anti-IGF2, dilution 1:100; R&D Systems, USA), while control sections were treated with an equal volume of PBS. Each biological replicate of the experimental and control groups was performed in triplicate. Following PBS washes, the sections were subjected to incubation with the secondary antibody (HRP-labeled rabbit anti-goat IgG, dilution ratio 1:200; Servicebio, Wuhan, China). Subsequently, sections were treated with DAB (Servicebio, Wuhan, China), followed by counterstaining, dehydration, mounting, and examined under microscope.

Cell assays: The isolated ovaries were washed with sterile PBS supplemented with 3% penicillin-streptomycin solution. The outer membrane, connective tissue, vascular network, and yolk of the follicles were carefully dissected and removed. The thoroughly cleaned follicular membranes were then minced into fine fragments. After washing and centrifugation, the precipitate was digested with 0.2% type II collagenase (Thermo Fisher, USA) in 37°C water for 30 min. The reaction was quenched by adding M199 complete medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (Gibco, USA). The cells were sequentially filtered through a 200μm stainless steel sieve and subjected to centrifugation (1000 rpm, 10 min, 4°C) to collect the cells. After cell determining density adjusted to 5×10^5 cells/mL^{-1}, the cells suspension was seeded into six-well culture plates and maintained at 37°C in a 5% CO_2 (Thermo Fisher Scientific, USA).

GC identification: HE staining and immunofluorescence were used to identify GCs. For HE staining, GCs were cultured for 24h, fixed in ethanol, stained with hematoxylin, differentiated with 1% hydrochloric alcohol, counterstained with eosin, and examined under an inverted light microscope (Olympus, Japan).

For immunofluorescence identification, GCs were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Beyotime, Shanghai, China), and blocked with 10% normal donkey serum (Abbkine, USA). The cells were then incubated with primary antibodies against follicle-stimulating hormone receptor (FSHR, ABclonal A3172) and luteinizing hormone receptor (LHR, Affinity AF9104). Subsequently, the cells were incubated with fluorescently labeled donkey anti-rabbit IgG and counterstained with DAPI (Beyotime, China). Fluorescent signals were visualized using a Zeiss confocal microscope (Oberkochen, Germany).

Cell counting kit-8 (CCK-8) assay: The proliferation of ovarian GCs treated with various concentrations of IGF2 (0, 50, 200 and 500ng/mL) for 48h was assessed by measuring the optical density (OD) at 450nm using the Cell Counting Kit-8 (Dojindo, Japan). The GC suspension was evenly distributed into 96-well plates, with six replicate wells per group. After 24h of culture, the CCK-8 reagent was added, followed by incubation for 2-4h. The OD values of each well were then measured at 450nm using a microplate reader.

5-ethynyl-2-deoxyuridine (EdU) assay: GCs treated with different concentrations of IGF2 for 48 h were stained using the commercially available BeyoClick™ EdU-488 Kit (Beyotime, Shanghai, China). The cells were divided into four groups: Control, IGF2-treated groups (50, 200, and 500ng/mL). After adding complete medium containing 50μM EdU working solution for 2h, the cells underwent fixation with 4% paraformaldehyde for 30 min. Permeabilization was then performed using 0.3% Triton X-100 in PBS for 10 min. After washing, Click Additive Solution and Hoechst 33342 reaction solution were added in turn. Cell morphology was documented using an Olympus inverted phase-contrast microscope (Tokyo, Japan) at a magnification of 100×.

Western blot analysis: To elucidate the role of IGF2 in modulating the PI3K-AKT signaling pathway during duck GC proliferation, we selected the optimal concentration of IGF2 (500 ng/mL) and the PI3K inhibitor LY294002 for treatment of GC. The expression levels of PI3K, AKT, p-PI3K, p-AKT, and IGF1R protein in the PI3K–AKT signaling pathway were detected using Western blot. Protein was extracted using the Total Protein Extraction Kit (Thermo Fisher Scientific, USA) and quantified with the BCA Protein Quantification Kit (Beyotime, Shanghai, China). Protein samples were separated by discontinuous SDS–PAGE with 8-12% resolving gel and 5% stacking gel at 80 V and 60 V respectively. Following electrophoresis and membrane transfer, the PVDF membrane was put into T-TBS supplemented with 5% BSA under ambient conditions for blocking. The membranes were separately incubated with PI3K, AKT, p-PI3K, p-AKT, IGF1R and β-actin (1:1000 AF6241, 1:500 AF6261, 1:500 AF3242, 1:500 AF0016, 1:1000 AF6125, Affbiotech and 1:2000 ab8226, Abcam). After washing with T-TBS, the membranes were incubated with goat anti-Mouse or goat anti-rabbit secondary antibodies (1:5000; 31160 and 31210, Thermo Fisher Scientific). Signals were developed by SuperSignal® West Dura Extended Duration Substrate (Thermo Fisher Pierce, Rockford, USA) and visualized on X-ray film. Band OD values were analyzed using ImageJ software, with three biological replicates (n = 3). The relative expression levels of target proteins were calculated as follows: target protein OD value / reference protein OD value, and results were expressed as mean ± SEM.

Transcriptome sequencing: The sequencing samples were divided into a control group (C-1, C-2, and C-3) and an IGF2-treated group (500 ng/mL; IGF2-1, IGF2-2, and IGF2-3). Total RNA was extracted from GCs using the Trizol Reagent (Invitrogen, CA, USA) following the manufacturer's protocol. RNA integrity was assessed using the Agilent 2100 Bioanalyzer system. Libraries that passed quality control were sequenced on the Illumina paired-end sequencing platform (PE150) with a read length of 150bp and an average insert size of 275bp. Data quality control was performed using fastp (version 0.19.7) with the following parameters: fastp -g -q 5 -u 50 -n 15 -l 150. Sequences were filtered to remove adapter-contaminated reads, reads containing undetermined bases (N), and reads in which more than 50% of bases had Qphred scores ≤ 5. The resulting clean reads were then aligned to the reference genome Anas platyrhynchos ("ncbi_anas_platyrhynchos_gcf_015476345_1_zju1_0") using HISAT2 software. Differential expression analysis was performed using DESeq2 software. Differentially expressed genes (DEGs) and pathways were identified based on the thresholds of $|\log_2$ (fold change)$| > 1$ and Padj<0.05. To further elucidated the biological significance of DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using the KOBAS platform (http://kobas.cbi.pku.edu.cn).

Quantitative real-time PCR (qRT-PCR): Total RNA was extracted from the heart, liver, spleen, lung, kidney, breast muscle, and ovary tissues of Jinding ducks using TRIzol reagent (Invitrogen, USA). RNA quality was assessed using a micro-spectrophotometer. First-strand cDNA was synthesized using the Reverse Transcription Premix Kit (AU341, TransGen Biotech, Beijing, China). For qPCR analysis, the *glyceraldehyde*-3-phosphate dehydrogenase gene served as the endogenous control. Primers for the target genes (Table 2) were designed using Primer Premier 6.0 and Beacon Designer 7.8 software and, synthesized commercially (Sunya Biotechnology Co., Ltd., Hangzhou, China). qRT-PCR amplification was carried out using the Premix qPCR Kit (AQ601, TransGen Biotech, Beijing, China). The PCR reaction mixture (20μL total volume) contained 10μL of 2×PerfectStart® Green qPCR SuperMix (Applied Biosystems, USA), 7.2μL sterile distilled water, 1.0μL cDNA template (2μg/μL), and 0.4μL of each upstream and downstream primers (10μmol/L). The thermal cycling conditions were as follows: initial denaturation at 94°C for 30 s, followed by 45 cycles of denaturation (94°C for 5 s) and annealing/extension (60°C for 30 s). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis: Quantitative data were expressed as the mean ± SEM from at least three independent experiments. Comparisons between two groups were analyzed using an independent sample t-test, and comparisons among multiple groups were performed using

one-way analysis of variance (ANOVA). Significance was set at P<0.05. Statistics were plotted using GraphPad Prism 9.5.1 software (GraphPad Software, La Jolla, CA, USA).

Table 2: *IGF2* primer sequence for qRT-PCR

Primers name	Sequence (5′ to 3′)	Gene ID
IGF2	F:GACAGGGGCTTCTACTTCAGTA R:AGTAGGTTTCCAGCAGAGCCA	XM_027457601.2
GADPH	F:GGAGCTGCCCAGAACATTATC R:GCAGGTCAGGTCCACGACA	XM_038180584.1
ANGPT2	GGAGCTGCCCAGAACATTATC GCAGGTCAGGTCCACGACA	XM_005016745.2
CCND3	GGCCTACTGTGACATGGAAAC CTCTCCAGCAGGATCACCAA	XM_038175947.2
FGF9	CTGCATGCTGCTGGCTTCCA AGCTGCTGAGGCGTGATGGA	XM_027445162.3
PGF	CGGTGTGCAGGATGCAGTAC GACTGACCCAGGTGGTCACTT	XM_038175265.2
GHR	GGAGATCTGGAATCGCAGCTTCT ACGCAGGAGGGTCTGAAGATGT	XM_038179881.2

RESULTS

Morphological characteristics of ovarian follicles in Jinding ducks: The ovaries of Jinding ducks at the peak laying stage exhibited well-developed follicles of various preovulatory follicles as depicted in Fig. 1. These follicles ranged from preovulatory to fully mature, displaying a gradual transition from white to yellow. Typically, 5–6 preovulatory follicles (F1–F5/F6) were observed, along with large yellow follicles (>8mm), small yellow follicles (SYF, 6–-8mm), large white follicles (3–-6mm), and small white follicles (1–-3mm). SYF were selected for GC culture.

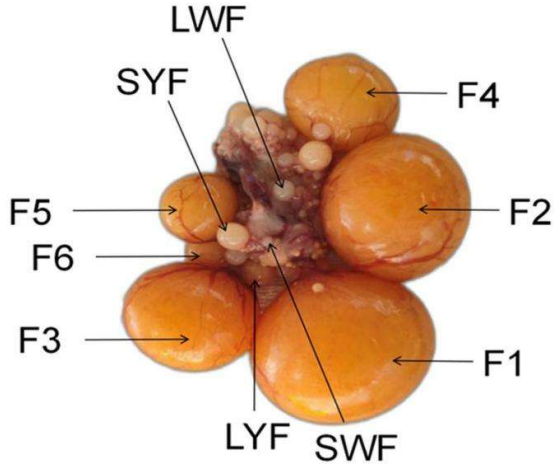

Fig. 1: Follicles at different developmental stages of the Jinding duck. Note: F1-F6 represented preovulatory follicles, ordered from largest to smallest diameter. LYF: large yellow follicles, SYF: small yellow follicles, LWF: large white follicles, SWF: small white follicles.

High expression of IGF2 mRNA in the ovary of Jinding ducks: The expression of IGF2 in the heart, liver, spleen, lung, kidney, breast muscle, and ovary of 300-day-old Jinding ducks was analyzed using qRT-PCR. The results showed that IGF2 expression in the ovary was significantly higher than in all other tissues (P<0.05), being 5.69-fold greater than in the liver, which had the second-highest expression level, and lowest in the breast muscle. No significant differences were detected among the remaining tissues (P>0.05) (Fig. 2).

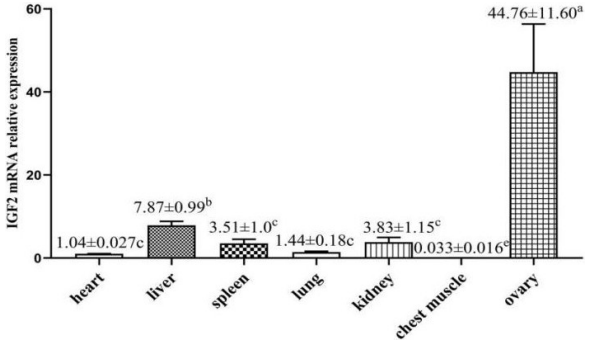

Fig. 2: The mRNA expression levels of IGF2 in different tissues. Note: Different lowercase letters indicated significant differences (P<0.05), while the same lowercase letter indicated no significant difference (P>0.05).

Distribution of IGF2 protein in duck follicles: The SYF, liver, and breast muscle tissues were subjected to HE staining and immunohistochemical analysis for IGF2 localization. HE staining revealed that the GC in the SYF were arranged in a monolayer columnar pattern (Fig. 3A-B). Immunohistochemical staining showed that the GCs and oocyte cytoplasm were weakly positive for IGF2, whereas the vascular endothelium exhibited strong positive staining (Fig. 3C-D). IGF2 was also positively expressed in duck hepatocytes (Fig. 3E-F), while the breast muscle showed week positivity (Fig. 3G-H). The protein expression feature was consistent with the mRNA expression levels.

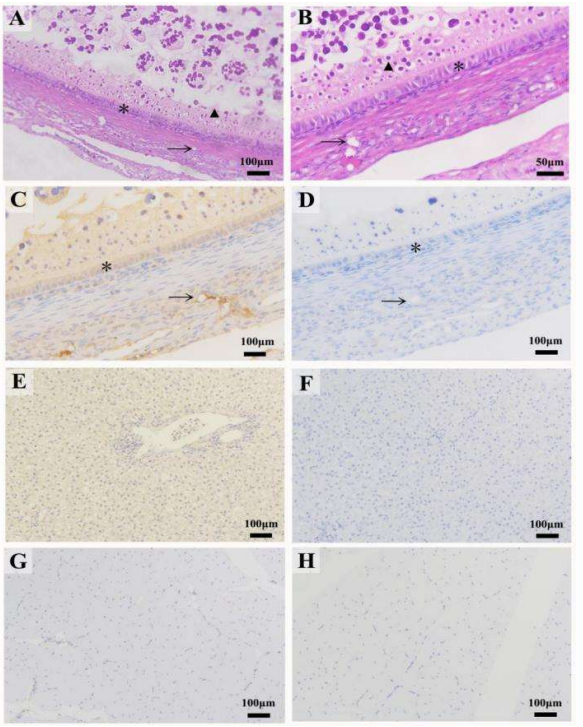

Fig. 3: The structure of granulosa cells and distribution of IGF2 protein. A and B, HE staining of small yellow follicle. Granulosa cells were monolayer columnar cells. C, immunohistochemistry reaction of IGF2 showed IGF2 distributed in granulosa cells (*), folliclar cytoplasm (▲) and the vascular endothelium (→). D, blank control for follicle. E, immunohistochemistry result of IGF2 in liver with weakly positive expression in hepatocytes. F, blank control for liver. G, immunohistochemistry result in chest muscle showed almost no expression of IGF2. H, blank control for chest muscle.

Isolation, culture, and characterization of GC: One week after primary cells were isolated and cultured, the cells exhibited epithelial cell-like, swirling growth, consistent with the characteristics of GC (Fig. 4). The morphology of the cells after HE staining was in line with the features of GC (Fig. 5A-C). Immunofluorescence analysis of FSHR and LHR indicated that over 90% of the isolated cells were GCs (Fig. 5E-J), ready for subsequent experiments.

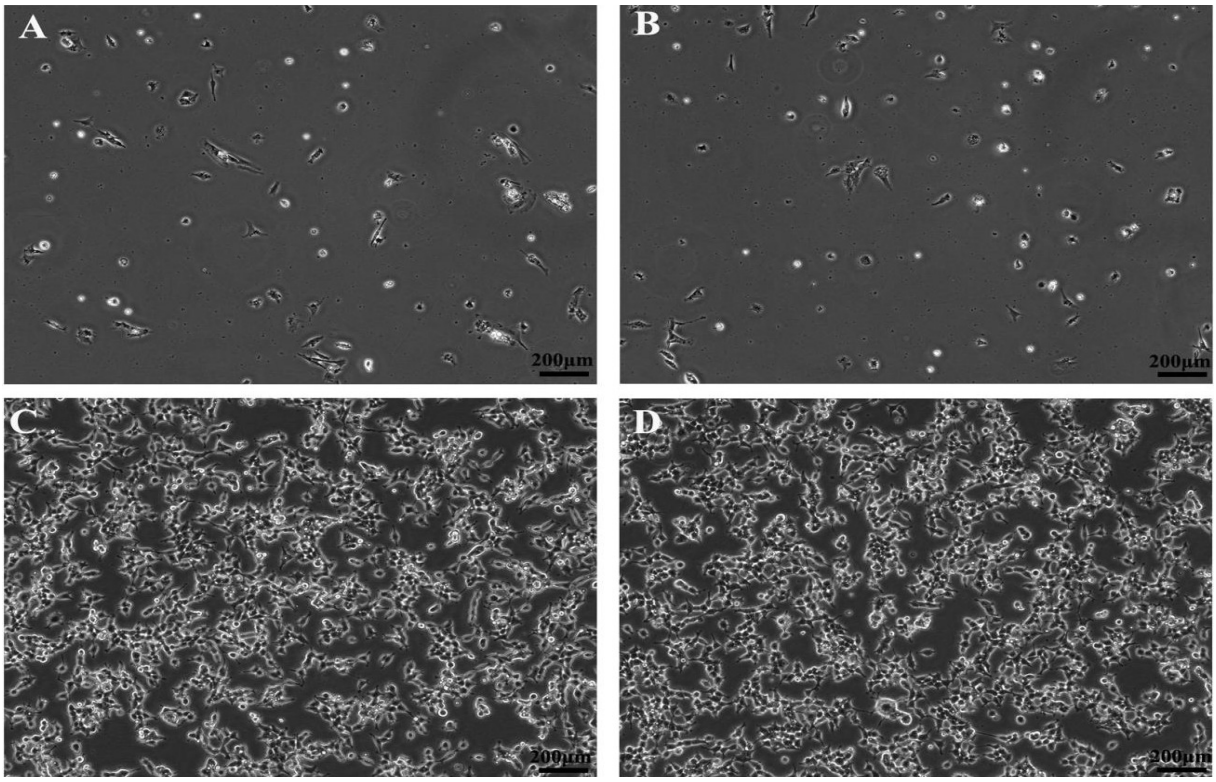

Fig. 4: Characterization of granulosa cell culture. A and C, cells were isolated and cultured for 16 h. B and D, cell morphology cultured for 1 week.

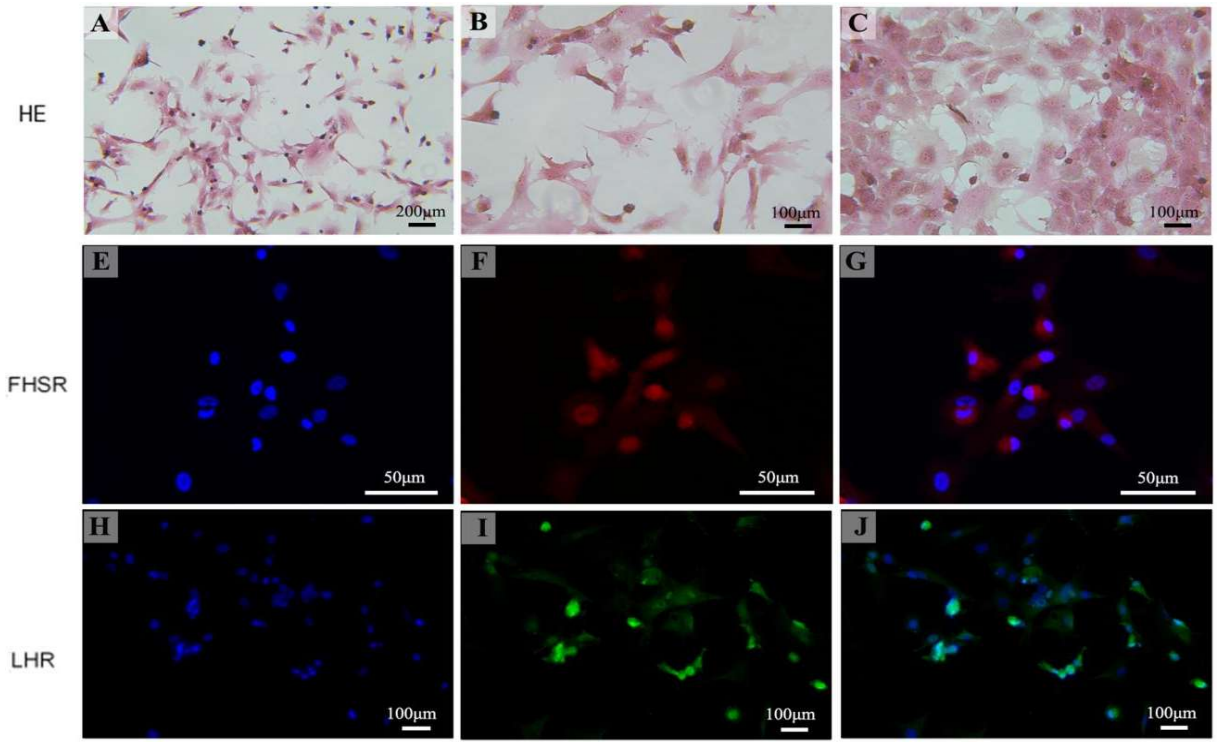

Fig. 5: HE and immunofluorescence identification of ovarian granulosa cells *in vitro*. A-C, HE staining of granulosa cells. D, nucleus stained with DAPI. E, FSHR-labeled granulosa cells. F, merge of nucleus and FSHR-labeled granulosa cells. G, nucleus stained with DAPI. H, LHR-labeled granulosa cells. I, merge nucleus and LHR-labeled granulosa cells.

IGF2 promotes proliferation of duck GC *in vitro*: GC were treated with various concentrations of IGF2 and divided into four groups. CCK-8 assays revealed that the IGF2 (500 ng/mL) group had the highest OD450 value compared to the control group, suggesting a significant effect on GC proliferation (P<0.01) (Fig. 6A). EdU assays further confirmed this finding, with a significant increase in EdU-positive cells in the IGF2 (500 ng/mL) group compared to the control (P<0.01) (Fig. 6B-C). Both CCK-8 and EdU assays demonstrated that IGF2 treatment promoted duck GC proliferation.

IGF2 promotes GC proliferation through the PI3K–AKT signaling pathway: After treatment with purified IGF2 and/or the PI3K inhibitor LY294002, protein expression at key nodes of the PI3K–AKT signaling pathway was examined. As shown in Fig. 7 and Table 3, IGF2-treated GC exhibited increased levels of p-PI3K, p-AKT and IGF1R proteins. In contrast, GCs treated with LY294002 alone showed reduced expression of p-AKT and p-PI3K. When both IGF2 and LY294002 were administered, the levels of p-AKT and p-PI3K increased compared with the LY294002-only group but remained lower than those in the IGF2-only group. These results indicate that IGF2 partially counteracted the inhibitory effect of LY294002 on the PI3K–AKT signaling pathway, thereby promoting the phosphorylation of PI3K and AKT and enhancing GC proliferation.

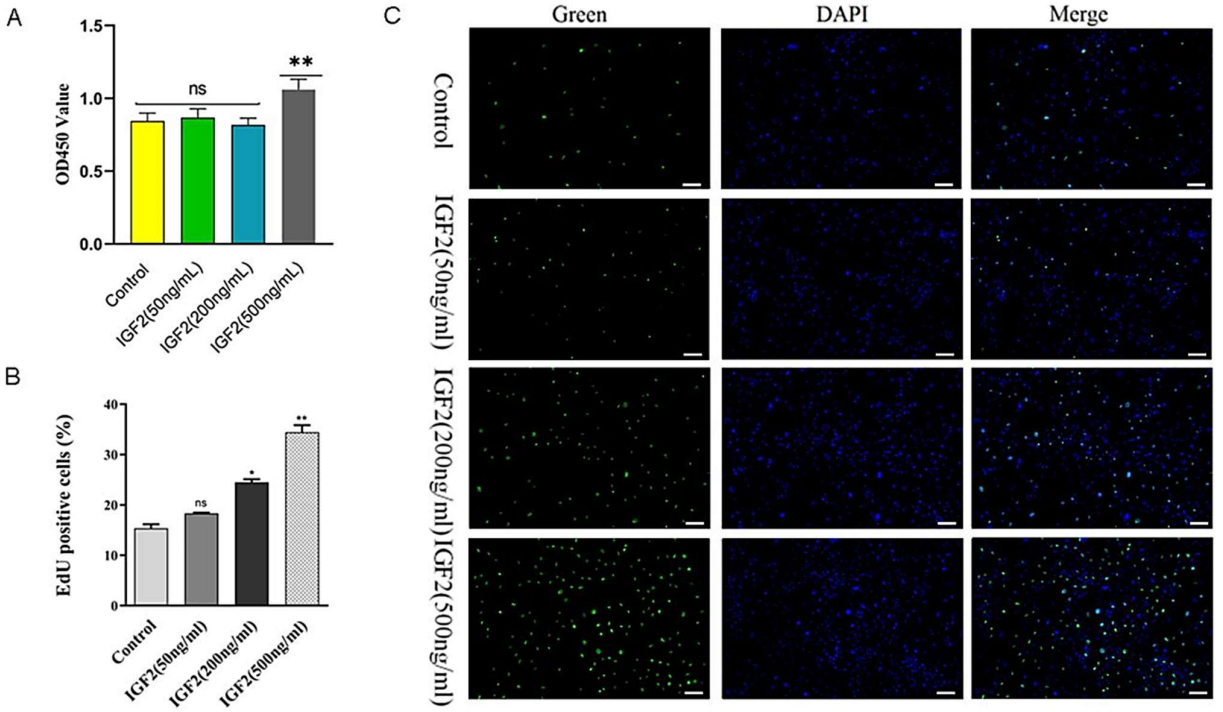

Fig. 6: CCK-8 and EdU assays for IGF2-induced proliferation of duck granulosa cells *in vitro*. A, OD450 value for the CCK-8 assays. B, percentage of EdU-stained positive cells. C, fluorescence images of EdU staining. ns indicated no significant difference (P>0.05), * indicated significant difference (P<0.05), ** indicated highly significant difference (P<0.01), Scale bar=100μm.

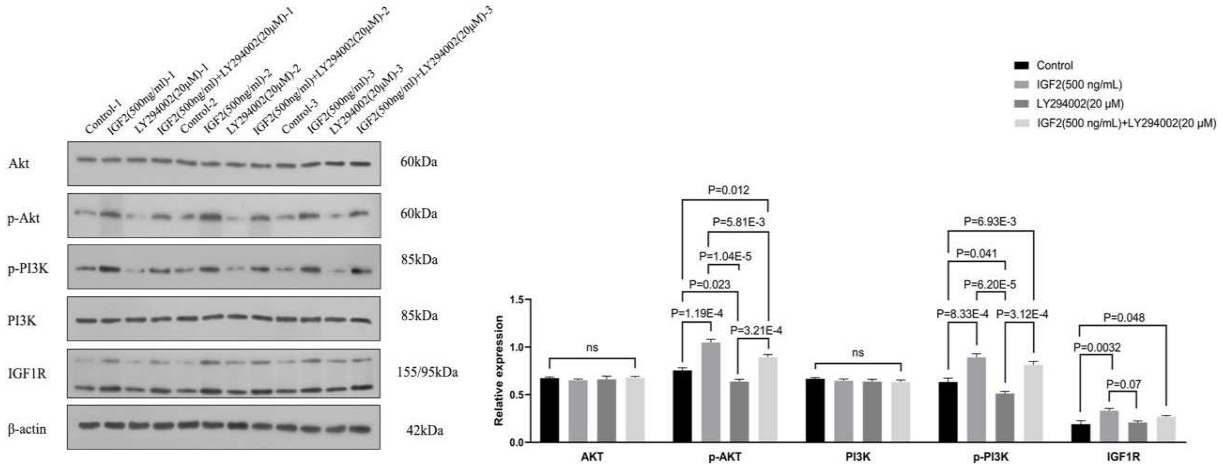

Fig. 7: Relative expression of PI3K-AKT pathway-related proteins in granulosa cells. A, detection of proteins by Western blot. B, the relative protein expression (mean±SEM), n=3. ns indicated no significant difference (P>0.05), * indicated significant difference (P<0.05), ** indicated highly significant difference (P<0.01).

Table 3: Relative expression level of PI3K-AKT pathway-related proteins in granulosa cells (mean ± SEM, n=3)

Group	AKT	p-AKT	PI3K	p-PI3K	IGF1R
Control	0.67±0.01	0.75±0.03	0.67±0.01	0.63±0.04	0.19±0.04
IGF2 (500ng/μl)	0.65±0.01	1.05±0.03	0.65±0.02	0.89±0.04	0.32±0.02
LY294002 (20ng/μl)	0.66±0.03	0.64±0.02	0.64±0.02	0.51±0.05	0.21±0.02
IGF2(500ng/μl)+LY294002(20ng/μl)	0.68±0.01	0.89±0.03	0.63±0.02	0.81±0.04	0.27±0.01

Transcriptomic changes in GCs induced by IGF2: Transcriptome sequencing was performed to observe RNA level changes in follicular GCs following IGF2 treatment. The results showed that 327 DEGs were screened in the IGF2-treated group, containing 120 upregulated and 207 downregulated genes compared with the control group. The volcano plot (Fig. 8A) and heatmap of DEGs (Fig. 8B) demonstrated reasonable clustering between the two groups, validating the accuracy of sequencing data. KEGG enrichment analysis demonstrated a substantial enrichment of DEGs in several key pathways, including focal adhesion, PI3K-AKT signaling pathway, extracellular matrix–receptor interaction, and neuroactive ligand-receptor interaction (Fig. 8C). These pathways played a vital role in cell growth, proliferation, signal transduction and extracellular matrix remodeling. The above enrichment characteristics suggested that IGF2 synergistically regulate the proliferation, adhesion and microenvironment adaptability of granulosa cells through multiple pathways, thereby affecting follicular development.

It is notable that, among the DEGs, 15 were found to be enriched within the PI3K-AKT pathway. Specifically, this set included 5 upregulated: cyclin 3 (*CCND3*), placental growth factor (*PGF*), G protein subunit gamma 4 (*GNG4*), laminin subunit gamma 2 (*LAMC2*) and fibroblast growth factor 10 (*FGF10*). In contrast, 10 genes were downregulated: platelet derived growth factor C (*PDGFC*), platelet derived growth factor D (*PDGFD*), fibroblast growth factor 9 (*FGF9*), growth hormone receptor (*GHR*), thrombospondin 2 (*THBS2*), collagen type IV alpha 5 chain (*COL4A5*), angiopoietin 2 (*ANGPT2*), collagen type IV alpha 4 chain (*COL4A4*), collagen type IV alpha 2 chain (*COL4A2*) and collagen type IV alpha 1 chain (*COL4A1*). Notably, *CCND3* exhibited up-regulation in the IGF2-treated group. This gene played a pivotal role in regulating the G1/S phase transition of the cell cycle. These findings implied that IGF2-induced proliferation of GC might be modulated through activation of the *CCND3*-mediated PI3K-AKT signal pathway. Consequently, this activation could promote GC growth and follicular development by regulating the cell cycle.

QRT–PCR Validation: In order to verify the reliability of RNA-seq results, qRT-PCR was used to detect the relative expression levels of 3 up-regulated genes and 2 down-regulated genes in ovarian granulosa cells of the experimental group and the control group. The results showed that the relative expression of up-regulated and down-regulated genes was consistent with the results of high-throughput sequencing (Fig. 9), indicating that the results of transcriptome analysis were reliable.

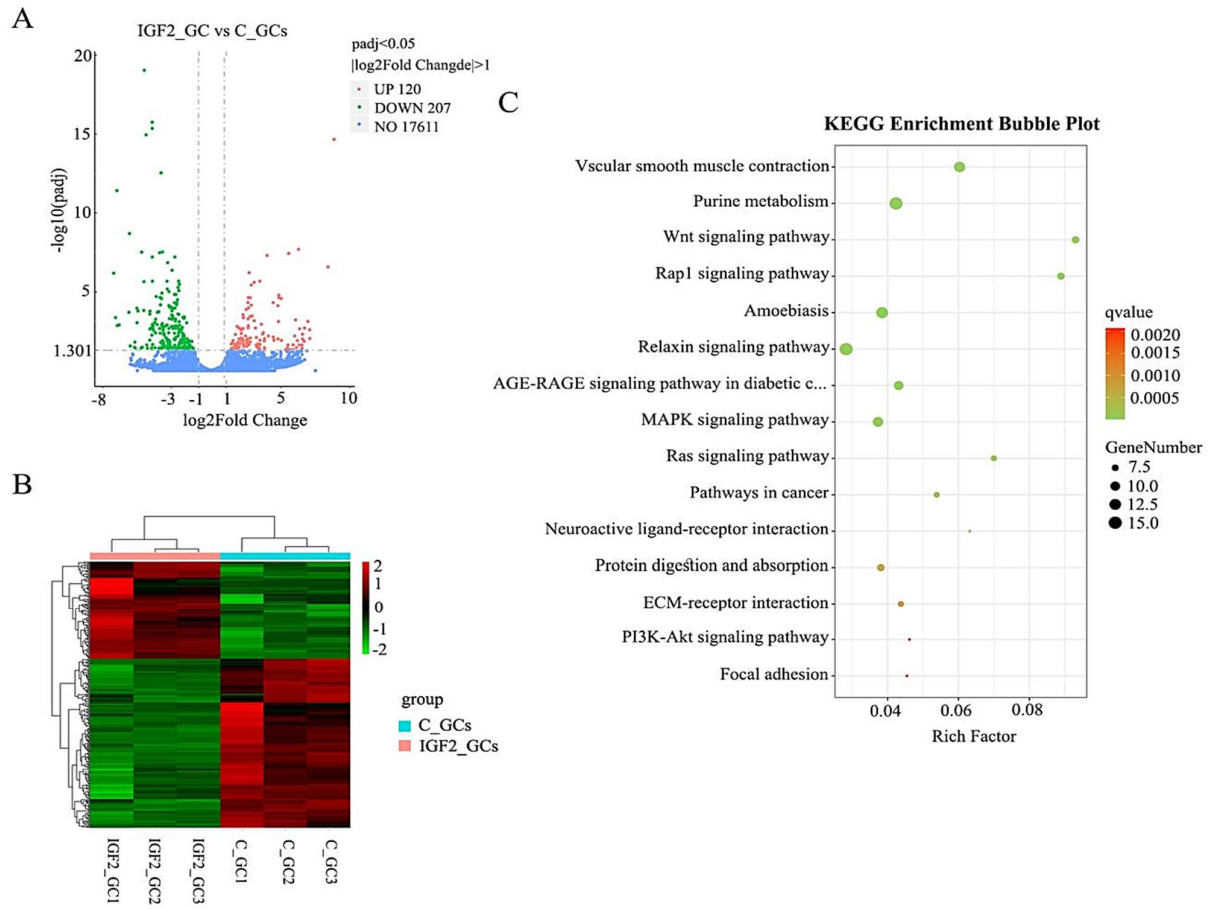

Fig. 8: Analysis of differentially expressed genes (DEG) in granulosa cells. A, the volcano map of DEG. B, the heat map of DEG. C, KEGG bubble plot.

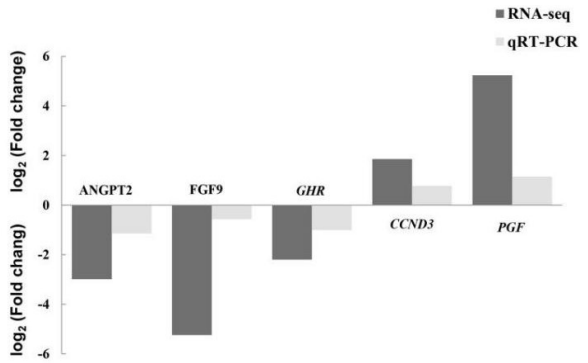

Fig. 9: qRT-PCR verification of differentially expressed genes (IGF2-treated group *vs* control group).

DISCUSSION

Granulosa cells played a central role in follicular development. However, the regulatory mechanism of IGF2 gene in granulosa cells of laying ducks remains unclear. GCs were pivotal functional cells within the three types that comprise ovarian follicles and were essential for ovarian development. They were located on the exterior of the hyaline, and facilitate material exchange and physiological interactions with the oocyte, while simultaneously nurturing and regulating its development (Luo *et al*., 2016). GC synthesized various growth factors and hormones that were indispensable for oocytes to modulate follicular growth, development, and maturation (Gong *et al*., 2019; Tan *et al*., 2021). While the function varied in ovarian GCs among avian species. The functional difference may be due to the specialization of follicular development patterns and endocrine regulatory networks in different species, such as definitive ovulation in chickens and ducks and uncertain ovulation in some birds (Yu *et al*., 2025). Especially, in geese, the role of granulosa cells was significantly greater than that of in follicular development (Liu *et al*., 2023). Despite numerous articles highlighting the importance of GC in follicular development, research on the key genes regulating GC in laying ducks remains scant. Through genome resequencing analysis comparing laying and broiler ducks, our research group identified *IGF2* as a potential candidate gene associated with laying duck reproduction. However, most existing studies on IGF2 in avian species have focused on chickens, geese, and other birds. Systematic reports on the functional verification and pathway analysis of IGF2 in laying ducks' granulosa cells have not been seen, which limited the comprehensive understanding of the reproductive regulation mechanism of laying ducks.

IGF2 exhibited a high expression pattern in the ovarian tissues and *granulosa cells* of various avian species. Insulin-like growth factors constitute a polypeptide family with growth-promoting and insulin-like effects. The IGF family (encompassing *IGF1*, *IGF2*, *IGF1R*, *IGF2R*, and *IGFBP*1-*6* played a fundamental role in follicular development, maturation, and ovulation (Ahmadi and Ohkubo, 2022). Among these, *IGF2* emerged as a critical regulator of embryonic growth, ovarian function, and tissue metabolism *IGF2* was widely expressed in Muscovy duck tissues, with the peak levels in the ovary (Ye *et al*., 2017). Similarly, *IGF2* elevated expression occurred in goose ovaries (Wang *et al*., 2023). High-yielding hens showed significantly greater follicular *IGF2* expression than low-yielding counterparts (Kim *et al*., 2004). Likewise, *IGF2* in ducks also showed high expression characteristics, and mainly located in GC and vascular endothelium. This suggested IGF2 vital role in regulating ovarian GC and follicular growth.

IGF2 stimulates the growth of GC and may be one of the determinants which controlled development of follicle in ducks. *IGF2* was found have an effect of GC proliferation in human dominant follicles (Baumgarten *et al*., 2015) and elevating survival rate of primate follicles and GC proliferation in macaques (Tkachenko *et al*., 2021). Moreover, *IGF2* was linked to egg-laying traits in chickens (Zhang *et al*., 2024). Yet little is known about the regulatory role of *IGF2* to duck ovaries and its effects on GCs are poorly documented. This study revealed that IGF2 could stimulate the proliferation of duck GC *in vitro*, and this indicated that IGF2 was possibly influence egg-production characteristics by affecting ovarian GC proliferation. It should be noted that this observation in agreement with past chickens' reports. In chickens, IGF2 also acted as an inhibitor of GC. On the condition of over-expression of *IGF2* in GC, the number of these cells increased, and the apoptosis in them was repressed. On the other hand, the apoptotic GC increased when *IGF2* was knocked out in chicken GC. All these findings pointed out that IGF2 stimulated the enlargement of chicken GC in combination with a rather suppressed rate of their apoptosis (Zhao *et al*., 2025). This implied that with nutritional control and the advanced breeding practices, it might be possible to breed duck with high levels of IGF2 meaning that the rate of egg production in duck would greatly increase.

IGF2 regulated the cell cycle by the PI3K/AKT/CCND3 signaling transduction pathway, thereby controlling the proliferation of duck ovarian granulosa cells. It was found in this study that supplementation of IGF2 into GC *in vitro* remarkably increased the levels of IGF1R, p-PI3K and p-AKT proteins and the expression of PI3K-AKT signaling pathway–related genes such as *CCND3*. This above observation suggested that IGF2 promoted cell cycle development through the PI3K–AKT signaling transduction and thus influence the proliferation of duck ovarian GC. It was very essential regulatory pathway for cell proliferation. The PI3K-AKT signaling pathway has been found to stimulate GC proliferation in sheep (Jia *et al*., 2024). In chickens, the engagement of this pathway improved GC proliferation as well (Deng *et al*., 2023). Similar results have been mentioned in human ovarian GC. The PI3K-AKT signaling pathway can be activated to inhibit excessive autophagy of GC, and enhance the function of ovaries (Dai *et al*., 2023). This pathway regulated ovarian dormancy and primordial follicle activation (Li *et al*., 2021). *IGF2* signaling may proceed by the way of IGF1R and finally transduce via the PI3K-AKT pathway to suppress human GC proliferation (Forbes *et al*., 2020). Over-expression of *IGF2* resulted in an increase levels of p-AKT, while *IGF2* knockout decreased it, and it was capable of controlling the proliferation and apoptosis of GC in chicken (Zhao *et al*., 2025).

The IGF2-mediated pro-proliferative effect CCND exhibited species differences. The Cyclin D family has three members, which are CCND1, CCND2, and CCND3.

CCND3 is one of the key proteins, used in cell cycle regulation. It majorly coordinated the G1 phase and played a significant part in the transition between G1 to S phase. *CCND3* regulated the cell cycle with attaching to cyclin-dependent kinases (CDK4 and CDK6) and induced phosphorylation, thus regulating downstream pathway and cell proliferation (Wang *et al*., 2019). Some past studies have suggested that IL-12 may increase the expression of *CCND3* through the activation of PI3K-AKT cascade which may in turn stimulate cell division (Yoo *et al*., 2002). Also, focal adhesion kinase (FAK) over-expression has been reported to activate this pathway which triggers an up-regulation of *CCND3* and an increase in the ability of cell proliferation (Yamamoto *et al*., 2003). GC proliferation was linked with *CCND3* in cattle (Shimizu *et al*., 2013). Equally, in geese, leptin has been indicated to be involved in the PI3K-AKT pathway, enhancing *CCND3* expression and GC proliferation (Wen *et al*., 2015). In this study, the induction of *IGF2* resulted in an up regulation of expression of the *CCND3* gene in duck ovarian GC. This observation implied that, *IGF2* can trigger PI3K–AKT signaling pathway, upregulate *CCND3*, affect the transition between G1/S phase and promote GC proliferation. It was important to note that this observation in ducks was contrary to what happened in chicken. The expression levels of the *CCND1* and *CCND2* in chicken went up following the over-expression of IGF2 (Zhao *et al*., 2025). This indicated that chickens and ducks shared consistency in the core pathway (PI3K/AKT), but there were species-specific differences in the regulation of downstream cyclins. When selecting candidate genes or molecular markers to assist in breeding poultry varieties with high reproductive performance, ducks should pay more attention to CCND3 in this pathway.

The IGF2-PI3K-AKT-CCND3 pathway played a positive role that leads to increasing the number of duck granulosa cells. While the cellular signaling network was complex and not independent of each other. Besides the PI3K-AKT pathway, MAPK/ERK was also a significant signaling pathway that controlled cell proliferation, differentiation, and survival (Sun *et al*., 2015). MAPK/ERK signaling pathway was an IGF1R downstream signaling pathway. IGF1R signaling may branch out to PI3K-AKT and MAPK/ERK. A decrease in MAPK expression affected the apoptosis of granulosa cells, thereby hindering their proliferation (Zhang *et al*., 2022). Activation of MAPK/ERK maintained hen granulosa cells in an undifferentiated state (Xu *et al*., 2018). Whether IGF2 concurrently regulated the MAPK/ERK pathway in duck granulosa cells, and whether there was functional complementarity or feedback regulation between the two pathways, remained important directions for future investigation. This will help construct a more comprehensive IGF2 signaling regulatory network.

Conclusions: This study demonstrated that IGF2 promoted the proliferation of duck GC in vitro and up-regulated the expression of key proteins and CCND3 mRNA in PI3K-AKT signaling pathway. It is clear that IGF2/PI3K/AKT/CCND3 is one of the key pathways that regulate granulosa cell proliferation and affect follicular development. It provided a theoretical basis and regulatory path for improving egg production performance and reproductive efficiency of ducks. However, this study still has limitations such as relatively small sample size and lack of *in vivo* verification. Future studies will need to construct *in vivo* experimental models and combine specific inhibitors or gene silencing methods to provide more solid experimental support for relevant conclusions.

Author contributions: Q C and L Z conducted the experiments, analyzed the data, and drafted the manuscript, and the two authors made equal contributions to this work. W S, L L, B Z, Q X and Z M contributed to animal experiments and cell culture. X L, C L, C P and Z Z were mainly responsible for RNA-seq analysis. Q H and N Z provided technical guidance. N Z were primarily responsible for experimental design and study supervision. All authors have reviewed and approved the final version of the manuscript.

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REFERENCES

Ahmadi S and Ohkubo T, 2022. Leptin promotes primordial follicle activation by regulating ovarian insulin-like growth factor system in chicken. Endocrinology 163:bqac112.

Dai W, Yang H, Xu B, *et al*., 2023. Human umbilical cord-derived mesenchymal stem cells alleviate excessive autophagy of ovarian granular cells through VEGFA/PI3K/AKT/mTOR pathway in premature ovarian failure rat model. Journal of Ovarian Research 16:198.

Deng X, Ning Z, Li L, *et al*., 2023. High expression of miR-22-3p in chicken hierarchical follicles promotes granulosa cell proliferation, steroidogenesis, and lipid metabolism via PTEN/PI3K/Akt/mTOR signaling pathway. International Journal of Biological Macromolecules 253:127415.

Forbes BE, Blyth AJ and Wit JM, 2020. Disorders of IGFs and IGF-1R signaling pathways. Molecular and Cellular Endocrinology 518:111035.

Gong Z, Yang J, Bai S, *et al*., 2019. MicroRNAs regulate granulosa cells apoptosis and follicular development: a review. Asian-Australasian Journal of Animal Sciences 33:1714.

Hensen K, Pook M, Sikut A, *et al*., 2020. Utilising FGF2, IGF2 and FSH in serum-free protocol for long-term in vitro cultivation of primary human granulosa cells. Molecular and Cellular Endocrinology 510:110816.

Hobeika E, Armouti M, Fierro MA, *et al*., 2020. Regulation of insulin-like growth factor 2 by oocyte-secreted factors in primary human granulosa cells. Journal of Clinical Endocrinology and Metabolism 105:327-335.

Irwin DA and Van Der Kraak G, 2012. Regulation and actions of insulin-like growth factors in the ovary of zebrafish (Danio rerio). General and Comparative Endocrinology 177:187-194.

Jia Y, Liu Y, Wang P, *et al*., 2024. NTRK2 promotes sheep granulosa cells proliferation and reproductive hormone secretion and activates the PI3K/AKT pathway. Animals 14:1465.

Kim M, Seo D and Ko Y, 2004. Relationship between egg productivity and insulin-like growth factor-I genotypes in Korean native Ogol chickens. Poultry Science 83:1203-1208.

Li L, Shi X, Shi Y, *et al*., 2021. The signaling pathways involved in ovarian follicle development. Frontiers in Physiology 12:730196.

Liu J, Dai S, Dai Z, *et al*., 2023. Transcriptome profiling of goose ovarian follicle granulosa cells reveals key regulatory networks for follicle selection. Animals 13:2132.

Lopez-Tello J, Yong HEJ, Sandovici I, *et al*., 2023. Fetal manipulation of maternal metabolism is a critical function of the imprinted IGF2 gene. Cell Metabolism 35:1195-1208.

Luo M, Li L, Xiao C, *et al*., 2016. Heat stress impairs mice granulosa cell function by diminishing steroid production and inducing apoptosis. Molecular and Cellular Biochemistry 412:81-90.

Ru M, Liang H, Ruan J, *et al*., 2024. Chicken ovarian follicular atresia: interaction network at organic, cellular, and molecular levels. Poultry Science 103:103893.

Scanes CG, 2022. Discontinuities in understanding follicular development, the ovulatory cycle and the oviposition cycles in the hen: advances, opportunities, slow downs and complete stops. Frontiers in Physiology 13:1023528.

Sélénou C, Brioude F, Giabicani E, *et al*., 2022. IGF2: development, genetic and epigenetic abnormalities. Cells 11:1886.

Shimizu T, Hirai Y and Miyamoto A, 2013. Expression of cyclins and cyclin-dependent kinase inhibitors in granulosa cells from bovine ovary. Reproduction in Domestic Animals 48:e65-e69.

Sun Y, Liu WZ, Liu T, *et al*., 2015. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. Journal of Receptors and Signal Transduction Research 35:600-604.

Tan Y, Huang X, Xu C, *et al*., 2024. Integrating genomics and transcriptomics to identify candidate genes for egg production in Taihe black-bone silky fowls (Gallus gallus domesticus). International Journal of Molecular Sciences 25:9373.

Tan Y, Xu X, Cao H, *et al*., 2021. Effect of age at first egg on reproduction performance and characterization of the hypothalamo-pituitary-gonadal axis in chickens. Poultry Science 100:101325.

Tkachenko OY, Wolf S, Lawson MS, *et al*., 2021. Insulin-like growth factor 2 is produced by antral follicles and promotes preantral follicle development in macaques. Biology of Reproduction 104:602-610.

Wang B, Wang Z, Han L, *et al*., 2019. Prognostic significance of cyclin D3 expression in malignancy patients: a meta-analysis. Cancer Cell International 19:1-12.

Wang C, Liu Y, Zhang Y, *et al*., 2023. Expression profile and the G63A mutation of IGF2 gene associated with growth traits in Zhedong-White goose. Animal Biotechnology 34:3261-3266.

Wang G, Deng H, Wang T, *et al*., 2024. Nutritional supplementation of breeding hens may promote embryonic development through the growth hormone-insulin like growth factor axis. Poultry Science 103:103945.

Wen R, Hu S, Xiao Q, *et al*., 2015. Leptin exerts proliferative and anti-apoptotic effects on goose granulosa cells through the PI3K/Akt/mTOR signaling pathway. Journal of Steroid Biochemistry and Molecular Biology 149:70-79.

Xin Q, Uyanga VA, Jiao H, *et al*., 2022. Insulin-like growth factor-1 is involved in the deteriorated performance of aged laying hens. Journal of Animal Science 100:skac286.

Xu R, Qin N, Xu X, *et al*., 2018. 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. Scientific Reports 8:9168.

Yamamoto D, Sonoda Y, Hasegawa M, *et al*., 2003. FAK overexpression upregulates cyclin D3 and enhances cell proliferation via the PKC and PI3-kinase-Akt pathways. Cellular Signalling 15:575-583.

Yang Y, Xu P, Liu J, *et al*., 2022. Constant light exposure in early life induces m6A-mediated inhibition of IGF gene family in the chicken. Journal of Animal Science 100:skac199.

Ye Q, Xu J, Gao X, *et al*., 2017. Associations of IGF2 and DRD2 polymorphisms with laying traits in Muscovy duck. PeerJ 5:e4083.

Yoo JK, Cho JH, Lee SW, *et al*., 2002. IL-12 provides proliferation and survival signals to murine CD4+ T cells through phosphatidylinositol 3-kinase/Akt signaling pathway. Journal of Immunology 169:3637-3643.

Yu R, Wei S, Amevor FK, *et al*., 2025. Niemann-Pick C2 regulates steroid hormone secretion and lipid deposition in chicken follicular granulosa cells. Poultry Science 104:105340.

Zhang G, Cui Z, Li J, *et al*., 2022. miR-122-5p regulates proliferation and apoptosis of chicken granulosa cells of hierarchal follicles by targeting MAPK3. Gene 824:146397.

Zhang W, Chen X, Nie R, *et al*., 2024. Single-cell transcriptomic analysis reveals regulative mechanisms of follicular selection and atresia in chicken granulosa cells. Food Research International 198:115368.

Zhao C, Hu B, Zeng X, *et al*., 2024. IGF2 promotes the differentiation of chicken embryonic myoblast by regulating mitochondrial remodeling. Journal of Cellular Physiology 239:e31351.

Zhao H, Sun G, Mu X, *et al*., 2023. Genome-wide selective signatures mining the candidate genes for egg laying in goose. BMC Genomics 24:750.

Zhao X, Wei Y, Li X, *et al*., 2025. Circular RNA WRNIP1 activates the PI3K-AKT and ERK1/2 signaling pathways by binding to miR-129-5p/IGF2 axis and facilitates ovarian follicle development in chickens. Poultry Science 104:104757.

Zhou J, Yu JZ, Zhu MY, *et al*., 2024. Genome-wide association analysis and genetic parameters for egg production traits in Peking ducks. Animals 14:1891.