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RESEARCH

Transcriptome analysis of *Schizothorax oconnori* (Cypriniformes: Cyprinidae) oocytes: The role of K^+ in promoting yolk globule fusion and regulating oocyte maturation

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Abstract *Schizothorax oconnori* (*S. oconnori*) is an economically important fish in Tibet. Oocyte maturation is a physiological process that is of great significance to reproduction and seed production in *S. oconnori*, yet little is currently known regarding the molecular mechanisms of oocyte development in this species. To identify candidate genes involved in reproduction of female fish, a combination of PacBio and Illumina HiSeq technologies was employed to provide deep coverage of the oocyte transcriptome. Transcriptome analysis revealed several candidate genes that are potentially involved in the regulation of oocyte maturation in *S. oconnori*, including *GIRK1*, *CHRM3*, *NPY2R*, *GABRA3*, *GnRH3*, *mGluR1α*, *GPER1*, *GDF9*, *HSP90*, and *ESR2*. Genes that are significantly expressed during oocyte maturation mainly contribute to the GPCR signaling pathway and the estrogen signaling pathway. Neurotransmitter (Ach, NPY, and GABA) and peptide hormone (GnRH3) binding to G protein-coupled receptors (GPCRs) frees G-protein βγ subunits to interact with the G protein-gated inward rectifier K^+ channel 1 (GIRK1). This process helps release K^+ from granulosa cells to maturing oocytes, allowing yolk globule fusion. This mechanism may play an important role in oocyte maturation in *S. oconnori*. In conclusion, this study provides a valuable basis for deciphering the reproductive system in *S. oconnori* during the oocyte maturation process.

Keywords *Schizothorax oconnori* · Transcriptome · Ovary maturation · Candidate genes · GABA

Introduction

Schizothorax oconnori (Fig. 1a), which belongs to the subfamily Schizothoracinae, the family Cyprinidae and the order Cypriniformes, is mainly distributed in the Yarlung Zangbo River in Tibet, China (Chen et al. 2013). In recent years, due to damming of rivers and overfishing, the natural resources available to schizothoracine fishes have gradually decreased. To sustain the development and utilization of schizothoracine fishes, artificial reproduction of these animals has been attempted by several institutions in Tibet (Bao-Shan et al. 2014). However, the spawning rates and

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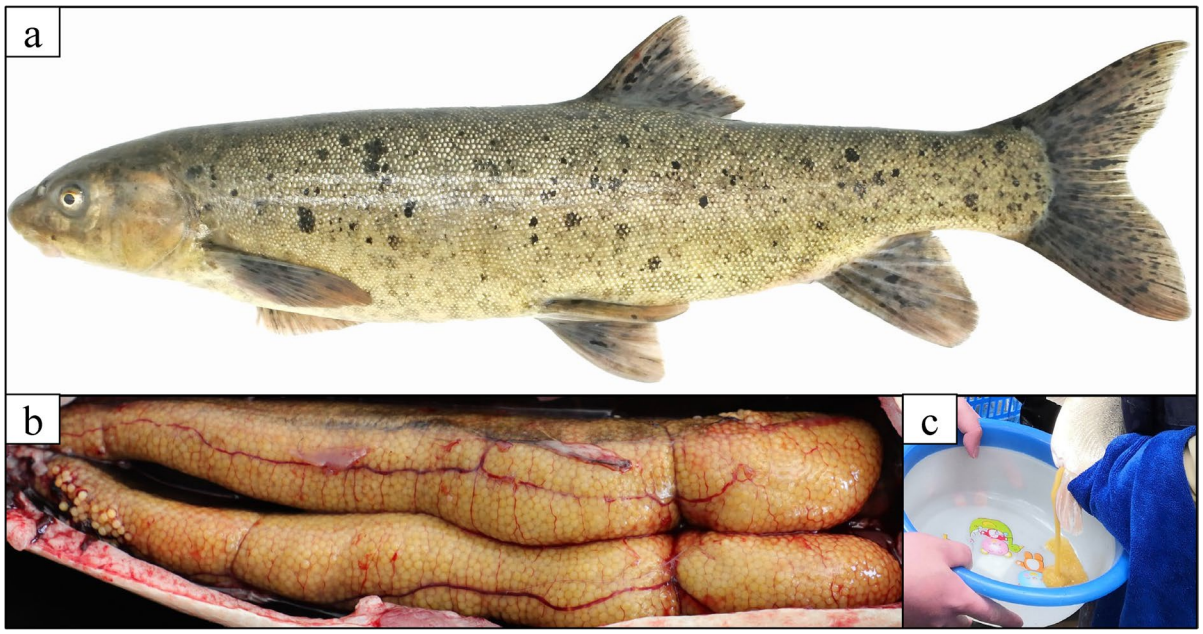

Fig. 1 Photographs of *Schizothorax oconnori*. **a** Outside view showing mean length and weight of 43.28 cm and 1356.43 g, respectively. **b** Two ovaries arrested in Phase IV and filled with oocytes; each ovary is enveloped by a thin ovarian membrane, and blood vessels are clearly visible within the ovaries. **c** Ovulated mature oocytes arrested in Phase V; these oocytes were obtained by lightly pressing the abdomen and were ovulated from the cloacal orifice during swimming

fertilization rates achieved are low due to the instability and low efficiency of artificial hormone-induced ovulation. This factor is currently seriously restricting the artificial maintenance of these species (Liang-Song 2011). Decreasing reproductive capacity and a declining growth rate are the major factors resulting in *S. oconnori* population decline. To overcome these problems, additional studies (including bioinformatics analyses) of *S. oconnori* oocyte maturation and reproduction should be conducted.

Oocyte development is a dynamic process that involves the coregulation of many cellular processes that support the development of gametes through the uptake of compounds and the modification of cell composition. Early studies of oocyte development focused on the regulation of endocrine and paracrine factors that affect fish ovary development (Sharpe et al. 1996). Recently, transcriptome profiling has provided a valuable method for studying the molecular mechanisms of ovary development in teleost fishes (Li et al. 2004; Lubzens et al. 2010; Tao et al. 2013; Chapman et al. 2014; Fan et al. 2014). Transcriptome analysis has been applied extensively to the study of gonads in many fishes, such as *Danio reri* (Li et al. 2004), *Morone saxatilis* (Chapman et al. 2014), *Paralichthys olivaceus* (Fan et al. 2014), and *Oreochromis niloticus* (Tao et al. 2013). In most female animals, including fishes, immature oocytes arrest in prophase I of meiosis, and during the period of arrest they grow through the accumulation of substances such as yolk and mRNAs. Hormonal stimulation allows oocytes to resume meiosis and proceed to metaphase II. These metaphase II-arrested oocytes are the final mature oocytes (Stitzel and Seydoux 2007; Suwa and Yamashita 2007). Numerous circulating endocrine and locally acting paracrine and autocrine factors regulate oocyte maturation. Maturation promoting factor (MPF) is a trigger for the initiation of oocyte maturation (Tanaka and Yamashita 1995). During vitellogenesis and oocyte maturation, the major regulators are Follicle stimulating hormone (FSH), Luteinizing hormone (LH), and Estradiol (E2) (Lubzens et al. 2010). In European sea bass, the expression of FSH and LH receptors is associated with the expression of GDF9 and BMP15 (Halm et al. 2008; Lubzens et al. 2010). GDF9 and BMP15 may play crucial roles during the growth of primary oocytes (Halm et al. 2008). In contrast, in zebrafish,

GDF9 is predominantly expressed in primary oocytes, while BMP15 transcript levels in oocytes do not change significantly at different developmental stages (Halm et al. 2008; Lubzens et al. 2010). ESR2, a receptor for E2, is essential for follicle maturation and ovulation (Khristi et al. 2018). However, analyses of the transcriptional characteristics of oocytes during oocyte maturation in *S. oconnori* have yet to be reported.

In *S. oconnori*, phase IV of oocyte maturation (Fig. 1b, late vitellogenic stage) is considered the appropriate period for artificial hormone-induced ovulation, and the oocyte matures in phase V (Fig. 1c, maturation stage). The membrane-bound yolk globules usually maintain their integrity throughout oocyte growth, and the yolk proteins stored in the globules initially accrue within crystalline inclusions. In this study, significant histological changes in the yolk globules of ovarian tissues were observed to occur between phase IV and phase V, indicating that ovarian tissues in phases IV and V are appropriate for studying the molecular basis of oocyte maturation in *S. oconnori.* The objective of the present study was to unravel the molecular mechanism of the oocyte maturation process in *S. oconnori*. To our knowledge, this is the first report on the transcriptomic profile of oocytes in schizothoracine fishes.

Materials and Methods

Animals and ethical considerations

Female *Schizothorax oconnori* (n = 72) were originally collected from the Yarlung Zangbo River drainage in Tibet, China.

Ethics approval and consent to participate

To ensure the welfare of the animals, all experiments and animal procedures were conducted strictly according to the protocols recommended by the Institutional Animal Care and Use Committee (IACUC) of Henan Agricultural University (permit number: 2017021) and the protocols supported by the regulations for animal experiments established by the Ministry of Science and Technology in China (2014). All the experiments and methods were designed with the aim of minimizing animal suffering. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Experimental treatment and sample collection

The oocytes of *S. oconnori* develop in synchrony. The fish were divided into two groups: those with ovaries in phase IV (n = 36) and those with ovaries in phase V (n = 36). Each group was then randomly divided into 3 subgroups (12 fish in each subgroup). Samples of ovarian tissue were collected from each subgroup. All animals were raised under the same acclimatized conditions (The composition of the diet is shown in Supplementary Table S1). The fish were euthanized using 100 mg/L MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA) and kept on ice before tissue collection. Each ovarian tissue sample was cut into pieces. A portion of the ovarian tissues was used for histological study, and the remainder of each sample was stored at -80 °C for later molecular analysis.

Histological study

Twelve samples representing the phase IV and phase V groups (6 biological replicates) were used for histological study. The samples (0.1 g tissue per sample) were fixed in Bouin's solution and transferred to 70% ethanol after 24 h. The pieces were then dehydrated, decolored, waxed in a dehydrator, and embedded in paraffin. Five-micrometer sections were prepared and stained with HE for histological observation.

RNA extraction, library preparation, and sequencing

The ovarian samples (a total of 3.0 g of ovarian tissue was taken from each oocyte sample in the phase IV and phase V groups) were pooled for total RNA extraction and the construction of PacBio sequencing libraries. Six libraries representing the phase IV and phase V groups (three replicates of each sample) were constructed for transcriptome sequencing (Illumina HiSeq). RNA concentration and RNA integrity number (RIN) were determined using agarose gel electrophoresis and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), respectively.

For PacBio sequencing, RNA samples were reverse-transcribed using the SMARTer® PCR cDNA Synthesis Kit (Clontech). PCR amplification

was performed using KAPA HiFi PCR Kits. BluePippin (Sage Scientific, Beverly, MA) was used for size selection of the sheared DNA. Large SMRTbell libraries were generated from the DNA products using SMRTbell Template Prep Kit 1.0. The SMRTbell libraries were sequenced on the PacBio RS II platform using P6-C4 chemistry. The libraries were also sequenced by paired-end sequencing on an Illumina HiSeq platform. Sequencing services were provided by Personal Biotechnology Co., Ltd., Shanghai, China.

Assembly of the transcriptome data obtained by PacBio and Illumina HiSeq sequencing

The raw sequencing data obtained using the PacBio RS II platform were filtered by SMRTLink (4.0) to obtain reads of insert (ROIs). The ROIs were classified according to whether the 5' primer/3' primer/polyA was complete and whether they were chimeric, and the full-length nonchimeric sequence was obtained. The long nonchimeric sequences were clustered using CD-HIT software (http://www.bioinformatics.org/cd-hit/) to obtain a nonredundant isoform for subsequent analysis.

The raw paired-end reads were cleaned by removing adaptor sequences, poly-N, and low-quality sequences. The obtained reads were mapped to the nonredundant isoform, and the unmapped reads were de novo assembled into contigs and transcripts using Trinity software (http://trinityrnaseq.sf.net) (Zhou et al. 2015). Finally, the assembly transcripts obtained from Illumina HiSeq and the nonredundant isoform obtained from PacBio-RSII were clustered by CD-HIT as unigenes for subsequent analysis.

Differentially expressed genes analysis

Unigenes were used for BLAST search and annotation against the NR, GO, KEGG, eggNOG and Swiss-Prot databases. The read counts were further normalized into FPKM (expected number of fragments per kilobase of transcript sequence per millions of base pairs sequenced) values. The FPKM values obtained for the two ovarian samples (oocytes in phase IV and phase V) with three replicates were pairwise compared, the fold changes in expression were calculated using RSEM software (version 1.2.7) (Li and Dewey 2011), and DEGs were identified using the DESeq R package (v1.18.0).

GO and KEGG enrichment analysis of DEGs

DEGs were also used in enrichment analysis through Gene Ontology (GO) using the GO-seq R package (Young et al. 2010). The adjusted *P* values of significantly substantiated GO terms were less than 0.05. KOBAS 2.0 software (http://kobas.cbi.pku.edu.cn/home.do) (Xie et al. 2011) was used to identify the KEGG pathways enriched with differentially expressed genes (FDR < 0.05).

qRT−PCR

qRT−PCR was used to determine the accuracy of the transcriptome sequencing results. Combined expression of the ACTB, CTSD, EEF1A and CTSZ genes was used as the internal control. The primers used to determine the transcription levels of these control genes have been described by Deloffre et al. (Deloffre et al. 2012). Primer pairs specific for the ten genes selected for SYBR green qRT−PCR were designed using Primer Premier 5.0 (Singh et al. 1998) (Table 1). The data are presented as relative transcript level based on the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

A completely randomized test design was used in the study. The significance of the difference between the means of the groups was determined by student's *t*-test. Differences with *P*-value < 0.05 (*) and *P*-value < 0.01 (**) were considered to be significant and extremely significant, respectively. The statistical calculations used in this study were IBM SPSS 24.0.

Results

Histological changes in S. oconnori oocytes

The membrane-bound yolk globules in developing *S. oconnori* oocytes usually maintain their integrity throughout oocyte growth, and the yolk proteins stored in the globules initially accrue within crystalline inclusions. The oocytes in phase IV (late vitellogenic stage) displayed significant yolk deposition.

Table 1 The primers for the twelve selected genes

Gene ID	Gene	Forward primer (5'-3')	Reverse primer(5'-3')
c27418_g1	GIRK1	TGATCTCATCATCAGTCTGGA	GTGGGGTCCATGTGAGTGGGAT
c192741_g1	CHRM3	TGCCCTTTTTGAGTTGTGCCA	ATTTGGAATAAGAGTCCAAGT
c50042_g1	NYP2	TGTATTTTTCTCCAATAACAAT	ATTTACAAGTTTAATTTCGTTA
c89088_g1	GABRA3	TCACAAAATTCCAACCTATCA	AATGGTCTCTGATCTCTTATC
c66366_g1	GnRH3	TGATCTGTCTCATCTCAGCTGT	TGCAGTGCCTGAACATGACTGA
c156721_g1	mGluR1α	TGCAGCAGCAGGAGCAGCACCT	TCAGATAGATGAGATGCAAGC
c69652_g1	GPER1	TGCGGAGTCGTGACAGCTACTAT	ACTCGTAGCATTCATCATGTTAC
c210986_g1	GDF9	TTGTTTACAATTCAGGTGTTAG	TGAGGACTCCGGCCTGAGCGTT
c103391_g1	HSP90	TGTTTGGCCAGATGCATTGAA	CCCTCAGTGCAGTTCATTTAT
c185766_g1	ESR2	TGTCAATGGCATCCTAGACAGT	TGCTGTCGAATCATTTCCTCAA

The zona radiata outside the cell membrane was thick, and the yolk granules had filled up the space outside the nucleus (Fig. 2a, c). In phase V (maturation stage), the nucleus could not be observed because the granular structures filled the entire cytoplasm. During the continued growth and maturation of the oocyte,

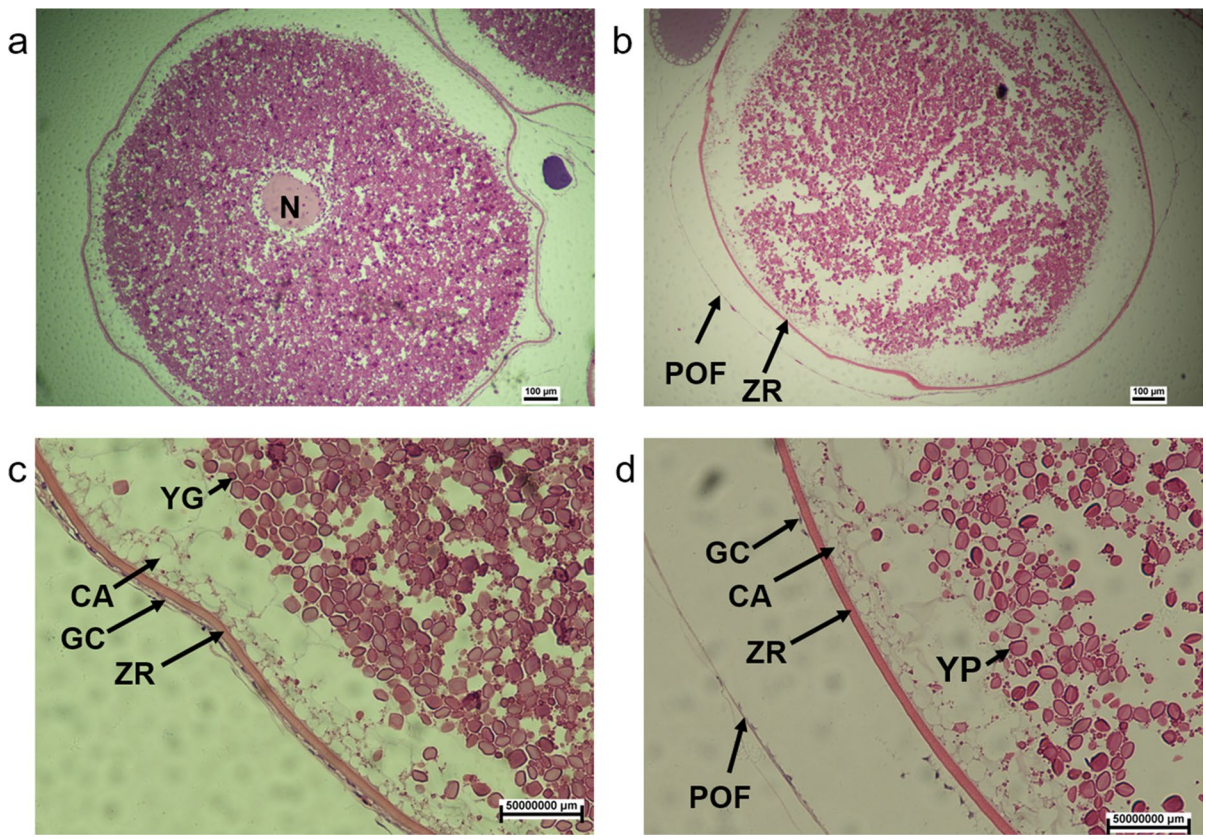

Fig. 2 Histology of oogenesis in *Schizothorax oconnori*. **a** and **c** Oocytes in phase IV showing nucleus, granulosa cells, zona radiata, cortical alveolus, and yolk granules. **b** and **d** Oocytes in phase V showing granulosa cells, zona radiata, cortical alveolus, yolk plate, and postovulatory follicles. N: nucleus; GC: granulosa cell; ZR: zona radiata; CA: cortical alveolus; YG: yolk granule; YP: yolk plate; POF: postovulatory follicle

the yolk granules fused centripetally to form yolk plates (Fig. 2b, d). The histological changes observed in the oocyte tissues from phase IV to phase V indicated that collection and transcriptome sequencing of oocyte tissues in phases IV and V would enable the elucidation of oocyte maturation in *S. oconnori*.

Combination of Pacbio sequencing and Illumina Hiseq sequencing

To analyze the changes in the transcriptome that occur during oocyte development in *S. oconnori*, oocyte tissues containing oocytes in phases IV and V were subjected to PacBio sequencing and Illumina HiSeq sequencing. Due to the short-read data generated by the Illumina platform, which led to inaccurate identification of full-length gene splice variants. Therefore, the PacBio RS II and Illumina HiSeq platform was used to sequence the transcriptomes of *S. oconnori* oocytes. A total of 460,927 reads of insert (ROIs) were obtained after filtering with SMRTLink (4.0). The mean read length of the inserts was 2,269 bp, and the mean read quality of the inserts was 0.98 (Supplementary Table S2). The accuracy of the ROI sequences obtained in this study reached a level that was sufficient for subsequent analysis, considering that the accuracy of the original data obtained using the PacBio platform was approximately 0.85 (Wagner et al. 2016). The number of full-length non-chimeric reads (reads that contained poly-A and the 5'- and 3'-ends of the primers) was 406,331 (88.16% of the total ROI reads). In total, 118,696 high-quality isoforms were identified (Table 2) as reference sequences that could be used to improve the assembly quality of the next-generation sequencing data.

To identify mRNAs that are differentially expressed in phases IV and V of oocyte development in *S. oconnori*, six Illumina HiSeq sequencing libraries were constructed from oocytes in each phase (Supplementary Table S3). The clean reads obtained by Illumina HiSeq sequencing were then mapped to the high-quality isoforms, and the unmapped reads were de novo assembled into contigs and transcripts using Trinity software. A total of 217,083 unigenes were obtained by assembling transcripts of Illumina HiSeq and the high-quality isoforms of PacBio using CD-HIT clustering. The maximal unigene length was 12,958 bp, with an average length of 1552.9 bp (N50: 2442 bp) and a GC% of 46.45% (Table 2). The unigenes were searched against the NR, GO, KEGG, eggNOG and Swiss-Prot databases using BLASTX with a cutoff E-value of $1e^{-5}$. Of the 217,083 unigenes, 159,034 (NR, 73.26%), 31,746 (GO, 14.62%) and 130,095 (Swiss-Prot, 59.93%) had significant matches. For further functional prediction and classifications, 148,525 (68.42%) unigenes were aligned to the eggNOG database; these genes clustered into 25 eggNOG categories ranging from A to Z by abbreviation of terms (Fig. 3). Among the functional classes identified, the largest and second largest clusters were those for general function prediction only (26,464, 17.82%) and signal transduction mechanisms (17.23%). The three smallest clusters were cell motility (0.07%), cell wall/membrane/envelope biogenesis (0.33%) and nuclear structure (0.52%) (Fig. 3). Pearson's correlation analysis of the samples showed that the correlation coefficients among the 3 replicates for each phase group were greater than 0.96 (Fig. 4a), indicating a high correlation. The correlation coefficients for the Phase IV

Table 2 Summary of de novo assembly of transcriptomes from *S. oconnori*

	Total Length (bp)	Sequence Number	Max. Length (bp)	Mean Length (bp)	N50 (bp)	N50 Sequence Number	GC %
Assembly of PacBio data	284287146	118696	12958	2395.09	2618	41807	47.11
Illumina unmapped reads assembly	67705202	115491	9282	586.238	777	24037	43.02
Combination of PacBio and Illumina data	337113399	217083	12958	1552.9	2442	51899	46.45

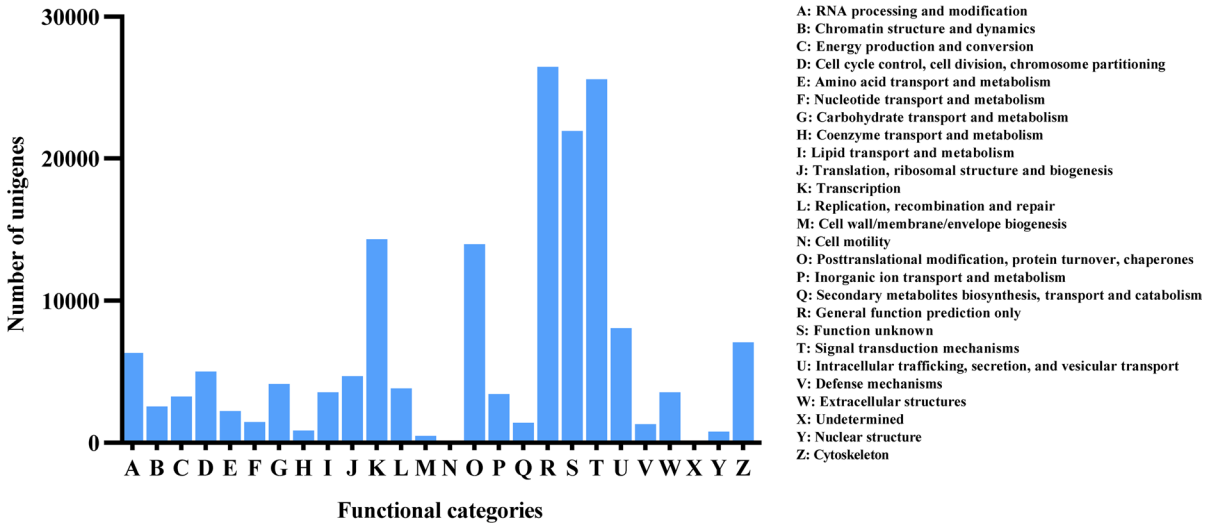

Fig. 3 EggNOG function classification of S. oconnori unigenes. A total of 148,525 unigenes were classified into 25 categories. The x-axis indicates the eggNOG category, and the y-axis indicates the number of unigenes

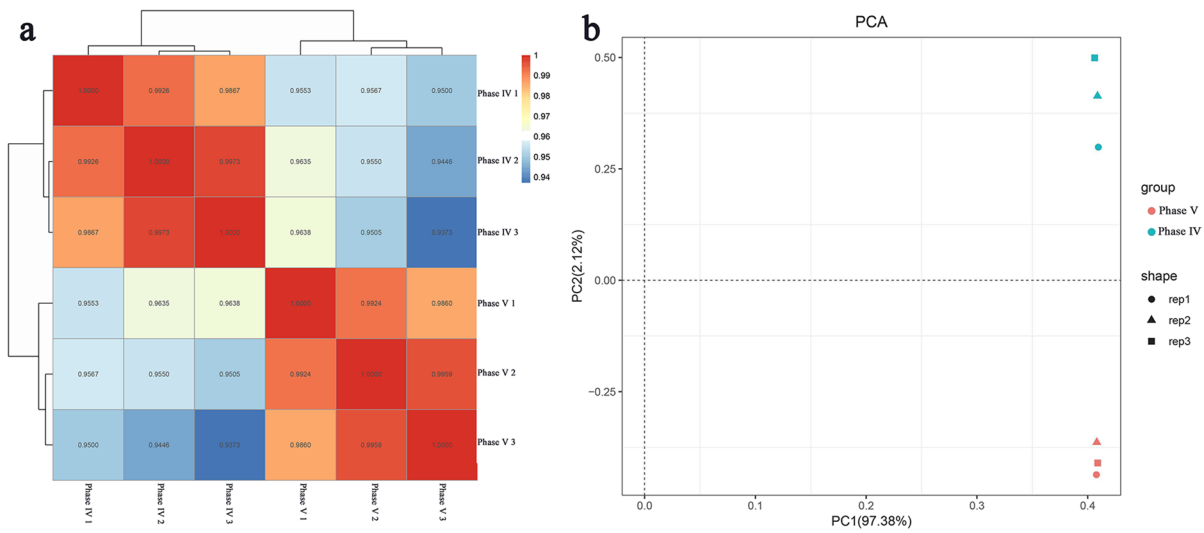

Fig. 4 Sample analysis of Phase IV and Phase V oocytes. **a** Correlation analysis of patterns of gene expression in Phase IV and Phase V. **b** Principal component analysis (PCA) of FPKM profiles in the two groups

vs. Phase V groups ranged from 0.9373 to 0.9638, suggesting a clear intergroup differential effect during oocyte development in *S. oconnori*. Consistent with this, PCA showed that the gene expression clusters in Phases IV and V were distinguishable (Fig. 4b), indicating the high reliability of our sequencing data.

Analysis of differentially expressed genes (DEGs)

To identify unigenes that are differentially expressed in phase IV and phase V oocytes, the expression of assembled unigenes was examined using the FPKM method. A total of 15,194 DEGs (those that were up- or downregulated at least twofold, with

P-value < 0.05) were found to be expressed at significantly different levels (Supplementary Table S4). Among them, 6,831 unigenes were upregulated and 8,363 (3.85%) were downregulated in phase IV vs. phase V oocytes (Fig. 5a). The volcano plots in Fig. 5b show the genes that are differentially expressed in phase IV and phase V oocytes of *S. oconnori*. Further clustering analysis showed that these DEGs clustered into 2 main groups (Fig. 5c). Compared with phase IV oocytes, phase V oocytes had more unigenes with high ratios of FPKM values. Remarkably, a large number of candidate genes related to oocyte maturation were identified and annotated among these DEGs; they included the genes encoding G protein-activated inward rectifier potassium channel 1 (*GIRK1*), muscarinic acetylcholine receptor M3 (*CHRM3*), neuropeptide Y receptor 2 (*NPY2R*), gamma-aminobutyric acid receptor subunit alpha-3 (*GABRA3*), gonadotropin-releasing hormone 3 (*GnRH3*), metabotropic glutamate receptor 1 alpha (*mGluR1α*), G protein- coupled estrogen receptor 1 (*GPER1*), growth differentiation factor-9 (*GDF9*), heat shock protein 90 (*HSP90*), and estrogen receptor beta (*ESR2*) (Table 3). As in the qRT−PCR results, the relative expression levels of these ten differentially expressed genes were consistent with the transcriptome sequencing results (Table 3).

Functional annotation of the DEGs

To understand the functions of the DEGs involved in oocyte development from phase IV to phase V, the identified DEGs were searched against the Gene Ontology and KEGG databases to determine GO functional and KEGG pathway enrichment. GO analysis showed that the DEGs were annotated to 4,190 GO terms. These DEGs were classified into three categories, namely, biological processes, molecular functions and cellular components. Most of the top 10 GO terms enriched in the regulated genes in each category were related to membrane and binding (Fig. 6a), suggesting that strategies for the regulation of the membrane and binding are modulated during oocyte development.

KEGG analysis revealed 2,683 DEGs that were assigned to 249 KEGG pathways. The top KEGG pathways enriched in the DEGs were neuroactive ligand−receptor interaction (71 DEGs, ko04080),

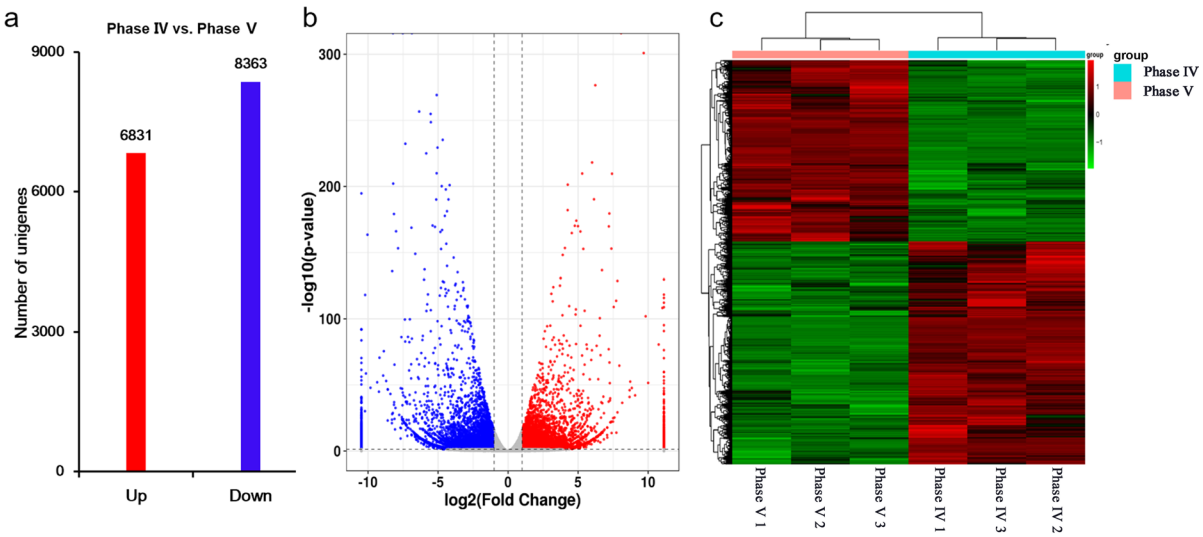

Fig. 5 Differences in unigene expression in phase IV and phase V ovaries. **a** The numbers of up- and downregulated genes in phase IV and phase V ovaries. **b** Volcano plots depicting genes that are differentially expressed in phases IV and V. The X-axis represents the level of differential expression, and the Y-axis represents the significance of gene expression differences. Genes with statistically significant differential expression are presented in red and blue; genes shown in gray exhibit no differential expression. (**c**) Heatmap showing the FPKM values of unigenes expressed in phase IV and phase V ovaries. The colors in the plot reflect the expression level, ranging from low (green) to high (red). The expression patterns of 15,194 unigenes were clustered into two main groups by hierarchical cluster analysis

Table 3 The candidate genes related to the ovary maturation with differently expressed genes between phase IV and phase V ovaries

Gene ID	Homologous function	Fold change		Log_2 fold change		
		FPKM	qRT-PCR (P5 ± SE/P4 ± SE)	FPKM	qRT-PCR	*P*-value
c27418_g1	G protein-activated inward rectifier potassium channel 1 (GIRK1)	6.28	5.82 (7.21 ± 0.61/1.24 ± 0.19)	2.65	2.54	0.005233
c192741_g1	muscarinic acetylcholine receptor M3 (CHRM3)	6.36	4.96 (9.07 ± 1.00/1.83 ± 0.24)	2.67	2.31	3.14E-06
c50042_g1	neuropeptide Y receptor 2 (NYP2)	+ ∞	39.40 (1.58 ± 0.44/0.04 ± 0.01)	+ ∞	5.30	1.63E-30
c89088_g1	Gamma-aminobutyric acid receptor subunit alpha-3 (GABRA3)	12.90	8.69 (9.13 ± 1.03/1.05 ± 0.25)	3.69	3.12	1.53E-07
c66366_g1	gonadotropin-releasing hormone 3 (GnRH3)	15.61	9.51 (2.38 ± 0.33/0.25 ± 0.10)	3.96	3.25	0.026069
c156721_g1	metabotropic glutamate receptor 1α (mGluR1α)	2.23	2.97 (14.19 ± 0.96/4.78 ± 0.94)	1.16	1.57	0.004186
c69652_g1	G-protein coupled estrogen receptor 1 (GPER1)	46.03	13.74 (13.46 ± 0.86/0.98 ± 0.30)	5.52	3.78	3.07E-05
c210986_g1	growth differentiation factor-9 (GDF9)	2.32	4.41 (68.80 ± 6.37/15.61 ± 2.48)	1.21	2.14	7.85E-11
c103391_g1	heat shock protein 90 (HSP90)	2.90	5.90 (44.99 ± 5.01/7.63 ± 2.03)	1.54	2.56	6.23E-13
c185766_g1	estrogen receptor beta (ESR2)	0.50	0.74 (8.25 ± 1.96/11.11 ± 3.25)	-1.01	-0.43	0.005655

Note: The padj value (padj < 0.05) represents the gene expressions from DEGs analysis were significant differences between phase IV and phase V ovaries

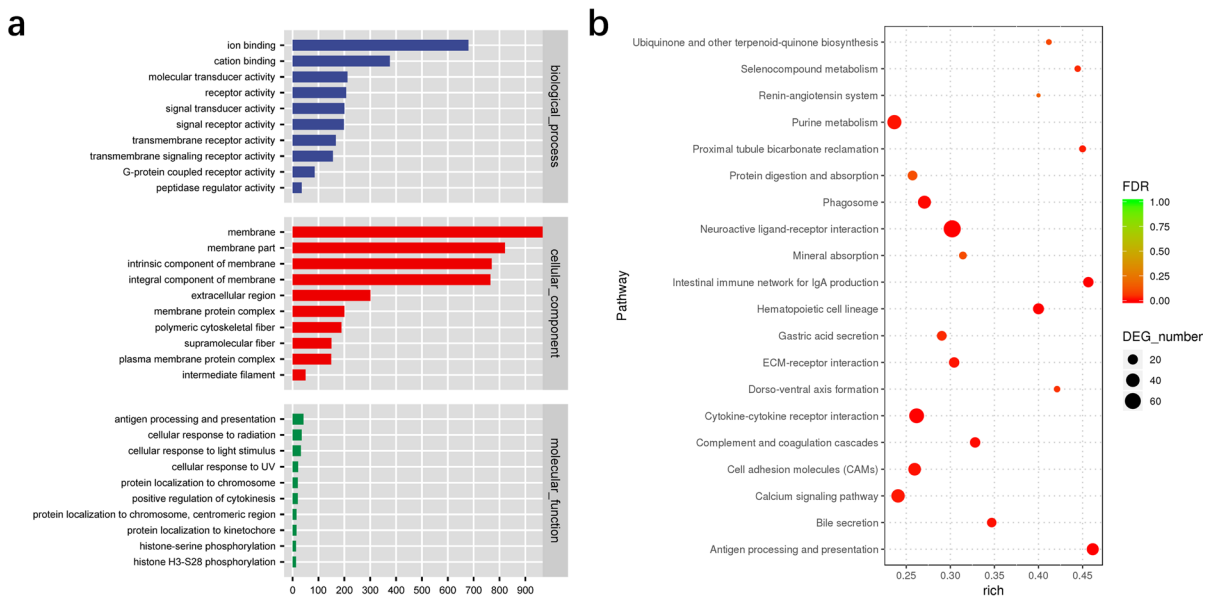

Fig. 6 Functional annotation of the DEGs. **a** GO term distribution for the categories biological process (red), cellular component (blue), and molecular function (green). The X-axis represents the number of unigenes. **b** KEGG pathway enrichment analysis. The X-axis indicates rich factors, and the Y-axis indicates KEGG pathway categories

cytokine−cytokine receptor interaction (50 DEGs, ko04060), purine metabolism (44 DEGs, ko00230), calcium signaling pathway (39 DEGs, ko04020), phagosome (36 DEGs, ko04145), and cell adhesion molecules (34 DEGs, ko04514) (Fig. 6b). A large number of the DEGs were involved in the PI3K-Akt signaling pathway (56 DEGs, ko04151) and the MAPK signaling pathway (41 DEGs, ko04010). Among them, the neuroactive ligand−receptor interaction (the GPCR signaling pathway) was enriched for the most differentially expressed oocyte maturation-related genes. In addition, the estrogen signaling pathway (5 upregulated genes, ko04915) and the GnRH signaling pathway (3 upregulated genes, ko04912) were also enriched for DEGs related to oocyte maturation. These results suggest that these pathways may play important roles in the oocyte maturation of *S. oconnori*.

Gene expression in the GPCR signaling pathway

G protein-coupled receptors (GPCRs) are cell-surface signal transmission receptor proteins that transfer messages related to extracellular conditions to intracellular effector molecules that participate in signaling or cellular physiological changes. Multiple genes associated with G protein signaling through the GPCR signaling pathway were significantly upregulated in phase V oocytes compared to phase IV oocytes (Fig. 7a). The upregulated genes belonging to Class A of GPCR signaling included *CHRM3*, *NPY2R*, and the genes encoding trace amine-associated receptor 4 (*TAAR4*), neuromedin U receptor 1 (*NMUR1*), neurotensin receptor type 1 (*NSTR1*), proteinase-activated receptor 1 (*F2R, PAR1*), prolactin releasing hormone receptor (*PRLHR, GPR10*), prostaglandin F2-alpha receptor (*PTGFR*), melatonin receptor type 1C (*MTNR1C*), leukotriene B4 receptor 1 (*LTB4R1*) and relaxin receptor 1 (*RXFP1*). The gene encoding GCGR in Class B of GPCR signaling was upregulated, as were *mGlu1α*, *GABRA3*, and *LEPR* in Class C. In contrast, the expression levels of dopamine receptor 4 (*DRD4*), histamine receptor H1 (*HRH1*), angiotensin II receptor 1 (*AGTR1*), C3a anaphylatoxin chemotactic receptor (*C3AR*), neuropeptide FF receptor 1 (*NPFFR1*), melanin-concentrating hormone receptor 1 (*MCHR1*), lysophosphatidic acid receptor 6 (*LPAR6*), cysteinyl leukotriene receptor 2 (*CYSLTR2*), parathyroid hormone 2 receptor (*PTH2R*), glutamate receptor ionotropic (*GRIN*), neuronal acetylcholine receptor subunit alpha-7 (*CHRNA7*), *P2RX2*, glutamate receptor 3 (*GRIA3*), glycine receptor subunit alpha-2 (*GLRA2*) and glucocorticoid receptor (*NR3C1*) were downregulated during oocyte maturation.

Gene expression in the estrogen signaling pathway

Estrogen signals are mediated through the estrogen receptor (*ER*), which functions as a transcription factor for target genes. Estrogen also regulates the functions of factors that produce nongenomic, rapid actions in cells through various mechanisms, including protein phosphorylation (Norman et al. 2004). During oocyte maturation in *S. oconnori*, the expression levels of G protein-activated inward rectifier potassium channel 1 (*GIRK1*), G protein-coupled estrogen receptor 1 (*GPER1, GPR30*), and metabotropic glutamate receptor 1 alpha (*mGluR1α*), all of which participate in the estrogen signaling pathway, were upregulated, whereas the expression levels of adenylate cyclase type 2 (*ADCY2*), estrogen receptor 2 (*ESR2*) and matrix metalloproteinase-2 (*MMP2*) were downregulated (Fig. 7b). A previous study proposed that neurotransmitter activation of a GPCR frees G-βγ, allowing it to interact with GIRK and causing the K^+ channel to open (Wickman and Clapham 1995; Dascal 2001; Nishida and MacKinnon 2002; Tabak et al. 2018).Above all, suggesting that appropriate enhancement of Ach, NPY, GABA and GnRH3 causes the corresponding GPCRs (CHRM3, NPY2R, GABR3A, and GnRH-R) to free G-βγ, allowing it to open GIRK1 (Fig. 7c).

Discussion

To explore the molecular mechanisms of oocyte maturation in *S. oconnori*, in this study we investigated the transcriptomes of phase IV and phase V oocytes using a combination of PacBio and Illumina HiSeq methods. Among the DEGs, GDF9 was significantly upregulated in phase V oocytes. It was previously reported that GDF9 expression is upregulated in the oocyte maturation stage compared with the late vitellogenesis stage in zebrafish (Halm et al. 2008; Lubzens et al. 2010). Thus, GDF9 likely plays a crucial role in the regulation of the oocyte maturation process in *S. oconnori*.

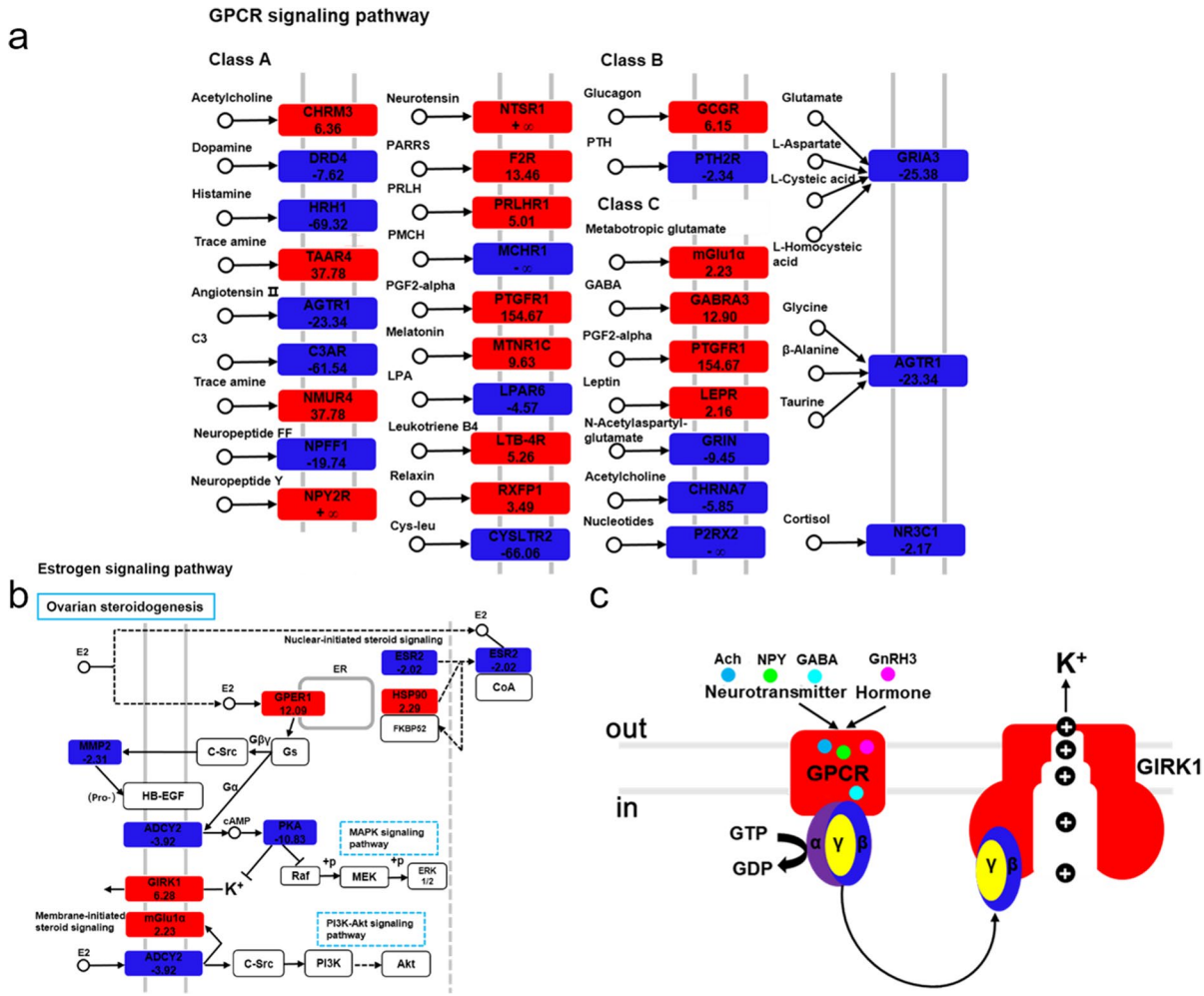

Fig. 7 Putative molecular mechanism of ovary maturation in *S. oconnori.* **a** DEGs involved in the GPCR signaling pathway. **b** DEGs involved in the estrogen signaling pathway. The upregulated unigenes are shown in red, and downregulated unigenes are shown in blue. The numbers in boxes indicate the fold change values. The fold change in the expression of upregulated genes was calculated as P5/P4. '-' indicates that the gene was downregulated; the fold change in expression of the downregulated genes was calculated as P4/P5. **c** Binding of neurotransmitters and hormones to G protein-coupled receptors (GPCRs) frees G-βγ, allowing it to interact with the G protein-gated inward rectifier K^+ channel 1 (GIRK1) and open the channel; this results in outflux of K^+ from granulosa cells to the maturing oocyte

ESR2 is well-known to play a role in oocyte development (Khristi et al. 2018), and ESR2 expression is stable between phase IV and phase V oocytes and that it may be regulated by E2 during oocyte development in *S. oconnori*. These results provide a valuable genomic resource for studying fish reproduction. HSP90 is required for the accumulation and activation of Mos, which is encoded by the *c-mos* protooncogene and induces *Xenopus* oocyte maturation (Fisher et al. 2000; Yamashita and Masakane 2000). In this study, HSP90 expression was significantly upregulated in phase V oocytes. These findings indicate that HSP90 induces oocyte maturation in *S. oconnori*.

Oocyte maturation is a physiological process that involves coregulation of many factors and in the modification of cell composition in a way that is significant for reproduction in *S. oconnori*. In phase IV oocytes, the yolk granules filled the space outside the nucleus, while in phase V oocytes, the yolk granules fused centripetally to form yolk plates (Fig. 2b). This phenomenon may be K^+-dependent (Fabra et al. 2006; Selman et al. 2010). The oocytes of marine teleosts

also undergo a significant increase in size due to rapid uptake of water during the maturation process (Wallace and Kelly 1981; Fabra et al. 2005; Selman et al. 2010; Watanabe and Kuo 2010). Yolk hydrolysis, however, is not the only mechanism underlying oocyte hydration, since the accumulation of ions such as K^+ and Cl^- during oocyte maturation may also contribute to water uptake (Finn et al. 2002; Fabra et al. 2005; Cerda et al. 2013). K^+ accumulation may play an important role during oocyte maturation in *S. oconnori*.

The transcriptome analysis conducted in this study showed that genes related to the GPCR signaling pathway and the estrogen signaling pathway were enriched among the DEGs during oocyte maturation. In agreement with our results, the GPCR signaling pathway has also been shown to be involved in testis development in the yellow catfish (Wu et al. 2015). In previous studies, it was proposed that neurotransmitter activation of a GPCR frees G-βγ, allowing it to interact with GIRK and resulting in the opening of K^+ channels (Wickman and Clapham 1995; Dascal 2001; Nishida and MacKinnon 2002; Tabak et al. 2018). Interestingly, the expression of GPCRs (*CHRM3, GABRA3, NPY2R, mGluR1α, F2R, RXFP1, TAAR4, NMUR1, NSTR1, GPR10, PTGFR, MTNR1C, LTB4R1, GCGR and LEPR*) and GIRK1 was significantly upregulated during oocyte maturation in *S. oconnori* (Fig. 7a, b). Moreover, we found that yolk granules fuse centripetally and eventually form yolk plates during oocyte maturation in a process that may be K^+-dependent (Fabra et al. 2006; Selman et al. 2010). K^+ presumably accumulates in granulosa cells, although the exact mechanism by which this occurs is unknown (Wallace et al. 1992; Cerda et al. 1993). In both wild and cultured teleosts, heterologous gap junctions (GJs), cell–cell contact structures that facilitate intercellular communication between the oocyte and overlying granulosa cells, form between the oocyte and granulosa cells. The passage of K^+ into the oocyte could be a passive process from the granulosa cells to the maturing oocyte through connecting GJs (Wallace et al. 1992; Cerda et al. 1993; Cheol et al. 2000; Xiaotian et al. 2000; Yoshizaki et al. 2001; Bolamba et al. 2003). It is evident that K^+ from granulosa cells translocates into oocytes during oocyte maturation (Wallace et al. 1992; Cheol et al. 2000; Xiaotian et al. 2000; Yoshizaki et al. 2001; Bolamba et al. 2003; Halm et al. 2008). Based on these findings, we propose a novel molecular mechanism concerning the activation of GPCRs in which they free G-βγ, allowing it to interact with GIRK1 and thus opening the K^+ channel. This causes more K^+ outflux from granulosa cells to the maturing oocyte, and the greater accumulation of K^+ in oocytes promotes yolk globule fusion, regulating oocyte maturation in *S. oconnori* (Fig. 7c).

GIRKs are regulated by neurotransmitters and hormones via G protein-coupled receptors (GPCRs) (Dascal 2001). GPCRs mediate the transduction of extracellular signals into complex intracellular responses (Farrants et al. 2018). The neurotransmitters that act through GPCRs mainly include acetylcholine (Ach), neuropeptide Y (NPY), γ-aminobutyric acid (GABA), dopamine (DA), and histamine (HA) (Kollonitsch et al. 1978; Briguglio et al. 2018; Farrants et al. 2018). The results of our study demonstrate that the expression of GPRCs (CHRM3, NPY2R, and GABR3A) was significantly upregulated during oocyte maturation in *S. oconnori*, while the expression of DRD4 and HRH1 was decreased (Fig. 7a). GnRH3 expression was also significantly increased. We propose that appropriate enhancement of Ach, NPY, GABA and GnRH3 causes the corresponding GPCRs (CHRM3, NPY2R, GABR3A and GnRH-R) to free G-βγ. This leads to opening of GIRK1 and release of K^+ from granulosa cells to the maturing oocyte. The greater accumulation of K^+ in oocytes promotes yolk globule fusion during oocyte maturation (Fig. 7a-c). This putative molecular mechanism provides a molecular basis for the development of rational management strategies for the sustainable utilization of *S. oconnori* resources. Further studies will focus on deciphering the mechanism through which schizothoracine GPCRs such as CHRM3, NPY2R, GABR3A, and GnRH-R interact with GIRK1 in *S. oconnori*. Additional functional studies and direct measurement of K^+ translocation through GIRK1 will be required to fully define the mechanism through which K^+ accumulation occurs in oocytes of *S. oconnori*. Finally, it should be noted that the sample size of this study is small and needs to be expanded in future studies.

Conclusion

In this work, gene expression profiles and biological pathways during oocyte maturation in *S. oconnori* were analyzed, and it was found that the GPCR signaling pathway and the estrogen signaling

pathway may play important roles in oocyte maturation in this species. The binding of neurotransmitters and peptide hormones to GPCRs frees G-βγ, allowing it to open GIRK1. This causes the release of more K^+ from granulosa cells to oocytes, resulting in yolk globule fusion. This study provides a valuable basis for deciphering the reproductive system in *S. oconnori* during the oocyte maturation process.

Authors' contributions JSZ, ML and ZCL conceived and designed the experiments. RTL and SJS performed the experiments. ZCL, JSZ and HFX analyzed the data. JSZ wrote the paper. All authors read and approved the final manuscript.

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Data Availability The datasets used and analysed during the current study have been deposited to Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number: PRJNA527715 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA527715) and PRJNA527627 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA527627).

Declarations

Ethical Approval All animal experiments were performed according to protocols and guidelines approved by the Institutional Animal Care and Use Committee of Tibet Academy of Agricultural and Animal Husbandry Sciences, China.

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

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