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Original Article

Comparative analyses of hypothalamus transcriptomes reveal fertility-, growth-, and immune-related genes and signal pathways in different ploidy cyprinid fish

Shengnan Li [1], Yi Zhou [1], Conghui Yang [1], Siyu Fan, Lu Huang, Tian Zhou, Qiubei Wang, Rurong Zhao, Chenchen Tang, Min Tao [*], Shaojun Liu [*]

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, Hunan Normal University, Changsha 410081, Hunan, PR China

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ABSTRACT

Triploid crucian carp (TCC) is obtained by hybridization of female diploid red crucian carp (*Carassius auratus* red var., RCC) and male allotetraploid hybrids. In this study, high-throughput sequencing was used to conduct the transcriptome analysis of the female hypothalamus of diploid RCC, diploid common carp (*Cyprinus carpio* L., CC) and TCC. The key functional expression genes of the hypothalamus were obtained through functional gene annotation and differential gene expression screening. A total of 71.56 G data and 47,572 genes were obtained through sequencing and genome mapping, respectively. The Fuzzy Analysis Clustering assigned the differentially expressed genes (DEGs) into eight groups, two of which, overdominance expression (6005, 12.62%) and underdominance expression (3849, 8.09%) in TCC were further studied. KEGG enrichment analysis showed that the DEGs in overdominance were mainly enriched in four pathways. The expression of several fertility-related genes was lower levels in TCC, whereas the expression of several growth-related genes and immune-related genes was higher levels in TCC. Besides, 15 DEGs were verified by quantitative real-time PCR (qPCR). The present study can provide a reference for breeding sterility, fast-growth, and disease-resistant varieties by distant hybridization.

1. Introduction

Distant hybridization refers to the cross between two different species or higher-ranking taxa, which can combine the biological characteristics of different species and results in genotypic and phenotypic changes of hybrid offspring [1,2]. Hybridization is a fundamental process in evolution, which leads to the merger of two or more different genomes to produce novel genotypes [3–6]. In the process of hybridization, the use of genetic complementarity between populations can produce heterosis. Heterosis is a complex biological phenomenon in which the hybrid offspring shows superior phenotypic characteristics than the parents, such as enhanced growth rate, development, and stress tolerance [7]. This phenomenon has been found in triploid fishes [8,9] and plants [10,11]. According to the principle of heterosis, the production efficiency of agricultural (livestock) products can be improved by the improvement and innovation of breeding methods. In contrast to heterosis utilization, the molecular mechanisms involved in this basic biological phenomenon are not yet clear. Heterosis is probably due to differences caused by qualitative or quantitative modification of gene expression [12–14]. Different regulatory factors and transcriptional networks would recombine in the hybrid and cause changes in gene expression [15–17]. Differential gene expression in parents and hybrids may be the cause of heterosis [18–21]. In contrast to its parents, a hybrid usually indicates expression levels of genes equal to the additivity (mid-parent), the high or low parent dominance (high or low parent), over-dominance (above the high parent), or underdominance (below the low parent) [22].

Triploidization is generally considered to be the most economical, practical, and effective method for large-scale production of sterile fishes. In recent years, the artificial induction of triploidy is the most popular ploidy manipulation in fish, because it caused sterility and mainly used to solve problems related to sexual maturity, such as

* Corresponding authors.
E-mail addresses: minmindiu@126.com (M. Tao), lsj@hunnu.edu.cn (S. Liu).
[1] These authors contributed equally to this work.

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decreased growth rates, increased incidence of diseases, and organoleptic deterioration of edible parts [23]. Triploidy has been artificially induced in many fishes [24–27]. Numerous studies have demonstrated that triploid individuals, with three sets of chromosomes, are generally sterile due to the unequal separation of chromosomes during meiosis, resulting in aneuploid gametes or retarded gonadal development [9,28].

Rapid growth is an important and highly anticipated feature that influences the profitability of fish production. The sterility of triploid individuals, resulting in more energy to be available for somatic growth since requirements for gametogenesis are partially (males) or totally (females) reduced, is considered to be able to significantly enhance the growth of triploid individuals when their diploid counterparts become mature [23]. However, an important aspect that should be considered before deciding to commercialize triploid fish is their resistance to diseases compared to that of diploid fish.

Teleost fishes, like their mammalian counterparts, possess innate immunity and adaptive immunity. Innate immunity is the first line of defense against pathogenic microorganisms and the main defense mechanism of fish against diseases. Innate immunity plays a very important role when vertebrates resist the invasion of external adverse conditions such as viruses and bacterial pathogenic microorganisms [29,30]. Fish diseases caused by viruses, bacteria, and parasites have become a major threat to the aquaculture industry, thus strong disease resistance has become one of the most important characteristics of economic aquaculture species. Measures are often taken to control, delay, or hinder sexual maturation and spawning to avoid threats to fish health during this time, for example, by inducing sterility through polyploidy [23]. However, to our best knowledge, the knowledge of understanding of the sterility, growth, and disease resistance mechanism of the sterile triploid fish is sparse.

The hypothalamus can be thought of as the coordinating center of the endocrine system and transmits precise signals to the pituitary gland, which then releases hormones that affect most of the endocrine systems in the body. The obtainment of the hypothalamus-pituitary axis is a major event in vertebrate evolution, which leads to neuroendocrine control of many complex functions, including reproduction, growth, stress, metabolism, and osmotic pressure [31]. Like mammals, fish growth and gonadal development are regulated by the hypothalamus-pituitary growth and hypothalamus-pituitary-gonad axes, respectively. Before sexual maturity, growth and development of individuals tend to show the effect of mutual promotion. However, when individuals reach sexual maturity, the individuals show a downward trend in the growth rate. During the gonadal development, the energy produced by fish metabolism is mainly consumed by the growth of sexual cells, while the growth rate of somatic cells is stopped or reduced.

Red crucian carp (*Carassius auratus* red var., RCC) is a variation of crucian carp belonging to Cyprinidae, Cyprininae, *Carassius*. Common carp (*Cyprinus carpio* L., CC), which belongs to Cyprinidae, Cyprininae, *Cyprinus*, is one of the paramount cyprinid species and accounts for 10% of freshwater aquaculture production worldwide [32]. In previous studies, bisexual fertile allotetraploid hybrids were produced by the intergeneric hybridization of female RCC and male CC. Subsequently, the allotetraploid hybrid lineage was generated by continuous self-crossing [2,33,34]. Triploid crucian carp (TCC) was obtained from the hybridization of female RCC and male allotetraploid hybrids, which was sterile and owned many merits, such as faster growth rate, stronger disease resistance and stress resistance, and high-quality meat [35]. TCC had two sets of RCC chromosome groups and one set of CC chromosomes. There are significant differences among different ploidy cyprinid fish not only in genetic composition but also in phenotype. In terms of reproductive characteristics, there are accumulating evidences showing that the ovaries of diploids within the breeding season contained many oocytes in stages II, III, and IV, and exhibited normal gonadal development [36–38]. However, the ovarian structures of triploids had obvious differences. The ovaries of triploids within the breeding season contained many small oogonium-like cells, only a few II-stage oocytes or even III-stage oocytes, no matured ova being found [35–37]. In terms of growth, the previous study indicated that compared with diploids, allotriploids grew faster [39]. The results of the mixed TCC and RCC cultured showed that the TCC grew 11.11% faster than RCC under the same conditions [40]. In terms of disease resistance, some studies demonstrated that triploids presented improved immunity compared with their diploid counterpart. For example, previous studies showed that the cell line from the caudal fin of TCC exhibited stronger antiviral resistance ability against SVCV than that of RCC [41], and the MAVS homologs of TCC had stronger antiviral activity than that of RCC [42]. Therefore, TCC and its original parents RCC and CC offer suitable models for the study of sterility, growth, and disease resistance mechanism.

RNA sequencing (RNA-seq) is a recently developed method for analyzing transcriptomes in a high-throughput and quantitative manner [43], with the advantages of highly accurate for quantifying expression levels and independence from genome sequence data [44]. In this study, we used RNA-seq to examine the transcriptome of mature female hypothalamic tissue from RCC, CC, and TCC. The purpose of this research was to understand the molecule bias and mechanism of fertility, growth, and disease resistance by identifying fertility-, growth-, and immune-related genes that were differentially expressed in TCC, relative to RCC and CC. Besides, the present study can provide a reference for breeding sterility, fast-growth, and disease-resistant varieties by distant hybridization.

2. Materials and methods

2.1. Ethics statement

All experiments, conducted in accordance with protocols from 2016 to 2018, were approved by the Animal Care Committee of Hunan Normal University. Approval was obtained for the Administration of Affairs Concerning Animal Experimentation guidelines from the Science and Technology Bureau of China. The methods were executed in accordance with the approved guidelines. Experimental individuals were fed in a pool with suitable illumination, water temperature, dissolved oxygen content, and adequate forage in the State Key Laboratory of Developmental Biology of Freshwater Fish located at Hunan Normal University, Changsha, China.

2.2. Sample collection and preparation

In May 2017, nine female individuals including three diploid RCC, three diploid CC, and three triploid TCC (14-month-old individuals) were selected at random from the State Key Laboratory of Developmental Biology of Freshwater Fish located at Hunan Normal University, Changsha, China. All individual fish was cultured in an open pool of 0.067-ha prior to tissue collection, and the ploidy of all individual fish was confirmed by flow cytometer (Cell Counter Analyzer, Partec, Germany) as described by Liu et al. [45]. The sex of each fish was determined by examining a gonad tissue slice according to microscopic histological examination as described by Long et al. [36]. The individuals were deeply anesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St Louis, MO, USA) before dissection to minimize suffering. The hypothalamic tissues excised from fish with different ploidy levels were snap-frozen in liquid nitrogen and then stored at −80 °C refrigerator for subsequent RNA isolation. We prepared three sets of hypothalamic RNA for each group for biological replication. Each set of RNA was a single RNA extracted from the hypothalamic tissue of three different individuals to provide a template for the construction of the RNA-Seq library (Supplementary Fig. S1).

2.3. RNA isolation, cDNA library construction, and sequencing

Total RNA was isolated using the Invitrogen TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's

protocol. All cDNA libraries were constructed and sequenced by the Novogene Corporation (Beijing, China) with an Illumina HiSeq™ 2500 platform using paired-end technology according to the manufacturer's instructions.

The clean reads were obtained by removing reads containing adapter sequences, low-quality reads with an average quality score of less than 20, and reads with ploy-N from raw reads using FastQC software (Babraham Bioinformatics) [46]. The clean data from the Illumina HiSeq™ 2500 were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and were available for download. Subsequently, these clean reads from each sample were mapped to the reference genome (RCC, http://bigd.big.ac.cn/gwh) [47] using HISAT software with the default parameters [48].

2.4. Identification of differentially expressed genes

For gene expression analysis, the expression level of each gene was quantified based on fragments per kilobase of transcript per million mapped reads (FPKM) values using HTSeq software (parameter: -m union) [49]. To avoid infinite values, a value of 1 was added to the FPKM value of each gene before a log10 transformation. Principal component analysis (PCA) was conducted using the R package of ggord [50]. The R package of DESeq2 [51] was used to identify differentially expressed genes (DEGs) with the adjusted *p* value for the FDR control (padj $\leq$0.05). Subsequently, these DEGs were used for following GO/KEGG enrichment analysis.

2.5. Cluster analysis and functional annotation

The normalized counts were clustered using the analysis of Fuzzy Analysis Clustering with default parameters except for setting 12 cluster classifications (c = 12). The software of Cluster3.0 [52] was used to generate heat maps that grouped DEGs according to *Z*-score values.

The functions of the DEGs were annotated using gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. GOseq software [53] was used to perform GO enrichment analysis, and KOBAS software [54] was utilized to test the statistical enrichment of DEGs in KEGG pathways. Corrected *P*-value <0.05 were thought to be significantly enriched.

2.6. Construction and topological analysis of protein-protein interaction network

The association between proteins can provide a clear point by grouping and organizing all the protein-coding genes in the genome that can be assembled into a large network [55]. The STRING database (https://string-db.org/) aims to assemble and evaluate the association information between proteins [56]. The protein-protein interaction networks were drawn using Cytoscape software [57]. The Cytoscape allows users to analyze and visualize networks of genes and compounds, and identify enriched pathways from expression profile data. ClueGO was used for data analysis. Using the ClueGO tool to quantitatively evaluate the degree of functional enrichment for a given cluster (calculate statistical significance) [58].

2.7. Quantitative real-time PCR analysis (qPCR)

To examine the reliability of the RNA-seq results, some DEGs involved in the fertility, growth, and disease resistance were selected for validation using quantitative real-time PCR (qPCR) on a Prism 7500 Sequence Detection System (Applied Biosystems, USA). The reaction mixture (10 μL) contained 5 μL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA), 1 μL of equally mixed cDNA (pooled from three hypothalami of the same type of fish), 0.5 μL of each primer, and 3 μL ddH_2O. The qPCR procedure was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was performed on biological replicates in quadruplicate to ensure the accuracy of the PCR results. The relative expression of each gene was normalized with *β-actin*, and the relative mRNA expression data were calculated using the $2^{-\Delta\Delta Ct}$ method [59]. All primers for qPCR were listed in Supplementary Table S1.

2.8. Statistical analysis

Unless mentioned otherwise, all qPCR data were expressed as the mean ± S.E.M. The statistical analysis was performed using SPSS 19.0 software (IBM, Chicago, IL, USA) to detect significant differences with One-Way Analysis of Variance (ANOVA) after Tukey's HSD Post-hoc test correction. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Transcriptome assembly

The ploidy of the RCC, CC, and TCC was determined by flow cytometry (Fig. 1A–C, Table 1). The ovarian microstructure of the RCC, CC, and TCC was determined by hematoxylin and eosin (HE) staining (Fig. 1D–F). A total of 492,156,094 paired-end reads, each with 150 bp long, were obtained using the Hiseq platform. All of the clean data were deposited in the NCBI SRA under accession numbers PRJNA649388, PRJNA649397, and PRJNA649440. After filtering out low-quality reads and adaptors, a total of 71.56 G data was obtained for further analysis (Supplementary Table S2). Through genome mapping (Supplementary Table S3), the hypothalamus transcriptome results showed that a total of 47,572 genes were obtained in RCC, CC, and TCC.

3.2. Differential expression analysis and gene annotation

The PCA result (Fig. 2A) and Pearson's correlation analysis (Fig. 2B) were used to show the correlation between biological replicates. As a result, we found high consistency among individuals of each fish. Besides, by comparing the gene expression levels of different ploidy fish of all genes, we found that TCC was closer to CC, which was consistent with the PCA result (Supplementary Fig. S2).

The significant DEGs between every two kinds of fish in RCC, CC, and TCC were identified with a padj value less than 0.05 and a $\log_2$(fold change) higher than 1 or lower than 1. In a comparative analysis, a total of 6375 DEGs were identified in CC compared with RCC, of which 2062 were up-regulated and 4313 were down-regulated. A total of 2311 DEGs were identified in RCC compared with TCC, of which 1149 were up-regulated and 1162 were down-regulated. A total of 11,477 DEGs were identified in CC compared with TCC, of which 4753 were up-regulated and 6724 were down-regulated (Fig. 3). Meanwhile, these results also indicated that these had nothing to do with ploidy, and there should be existed a non-additive expression. KEGG pathway enrichment was assessed to the DEGs. When comparing CC and RCC, the first ten enriched pathways were NOD-like receptor signaling pathway, Nitrogen metabolism, Carbon metabolism, Biosynthesis of amino acids, Pyruvate metabolism, Herpes simplex infection, Adipocytokine signaling pathway, Metabolism of xenobiotics by cytochrome P450, Glycosphingolipid biosynthesis - globo series, Toll-like receptor signaling pathway. These enriched pathways function in immune-related signaling pathways, carbon-nitrogen metabolism, and amino acids synthesis, which might confer the differences in the developmental process of hypothalamic tissues between CC and RCC (Supplementary Fig. S3A). When comparing CC and TCC, the first ten enriched pathways were Oxidative phosphorylation, Carbon metabolism, Citrate cycle (TCA cycle), NOD-like receptor signaling pathway, Propanoate metabolism, Pyruvate metabolism, Biosynthesis of amino acids, Cardiac muscle contraction, Spliceosome, Nitrogen metabolism. These enriched pathways had functions in energy metabolism, amino acids synthesis, and

cardiac muscle contraction, which might indicate the differences in the developmental process of hypothalamic tissues between CC and TCC (Supplementary Fig. S3B). When comparing RCC and TCC, these DEGs were enriched in Vascular smooth muscle contraction, GnRH signaling pathway, Apoptosis, Wnt signaling pathway, MAPK signaling pathway (Supplementary Fig. S3C).

3.3. Fuzzy analysis clustering

Through Fuzzy Analysis Clustering, the expression genes of RCC, CC, and TCC were divided into eight subclusters (Fig. 3), of which two subclusters were underdominance (3849, 8.09%) and overdominance (6005, 12.62%) expression patterns. Subsequently, the heatmaps which grouped DEGs according to *Z*-score values were generated (Fig. 4). The DEGs showed underdominance patterns were expressed repression in Cluster 3, however, these genes showed overdominance patterns were expressed activation in Cluster 4.

3.4. Function annotation of DEGs in underdominance

A total of 3849 DEGs showed underdominance patterns were classified into diverse functional categories by GO and were associated with KEGG pathway mapping. When analyzing the GO terms, we found that many of the DEGs were enriched in biological processes such as peptide biosynthetic process, amide biosynthetic process, and peptide metabolic process (Supplementary Fig. S4). When analyzing the KEGG pathways, we found that at least three pathways were involved in reproductive biology, including GnRH signaling pathway (8 DEGs), Steroid hormone biosynthesis (16 DEGs), and Estrogen signaling pathway (18 DEGs). NF-kappa B signaling pathway (47 DEGs) and Phagosome (48 DEGs), which involved in disease resistance, were enriched. Ras signaling pathway (36 DEGs) and MAPK signaling pathway (58 DEGs) related to growth development were also enriched (Supplementary Fig. S5).

3.5. Function annotation of DEGs in overdominance

A total of 6005 DEGs showed overdominance patterns were classified into diverse functional categories by GO and were associated with KEGG pathway mapping. When analyzing the GO terms, we found that many of the DEGs, which involved in reproductive biology, were enriched in biological processes such as steroid hormone mediated signaling pathway, response to steroid hormone, and cellular response to steroid hormone stimulus (Supplementary Fig. S6). When analyzing the KEGG pathways, we found that Steroid biosynthesis (12 DEGs), MAPK signaling pathway (128 DEGs), and Phagosome (20 DEGs) related to reproductive biology, growth development, and disease resistance, was enriched, respectively (Supplementary Fig. S7).

3.6. Topological analysis of protein-protein interaction network among RCC, CC, and TCC

Protein-protein Interaction Network was assessed to the DEGs of underdominance and overdominance expression patterns (Fig. 5). The results showed that the DEGs of underdominance patterns were mainly enriched in Ribosome, Calcium signaling pathway, and Neuroactive ligand-receptor interaction and the DEGs of overdominance patterns were mainly enriched in MAPK signaling pathway, Calcium signaling pathway, Neuroactive ligand-receptor interaction, and Phagosome. The phagosome pathway was significantly higher in TCC than in the original parents, which were related to the disease and stress resistance function of TCC. Studies showed that some genes involved in cell phagocytosis of TCC exhibited overdominance expression pattern, which was related to the enhancement of the autoimmunity of TCC.

3.7. Validation of DEGs by qPCR

To verify the quality of RNA-seq data and the reliability of genes

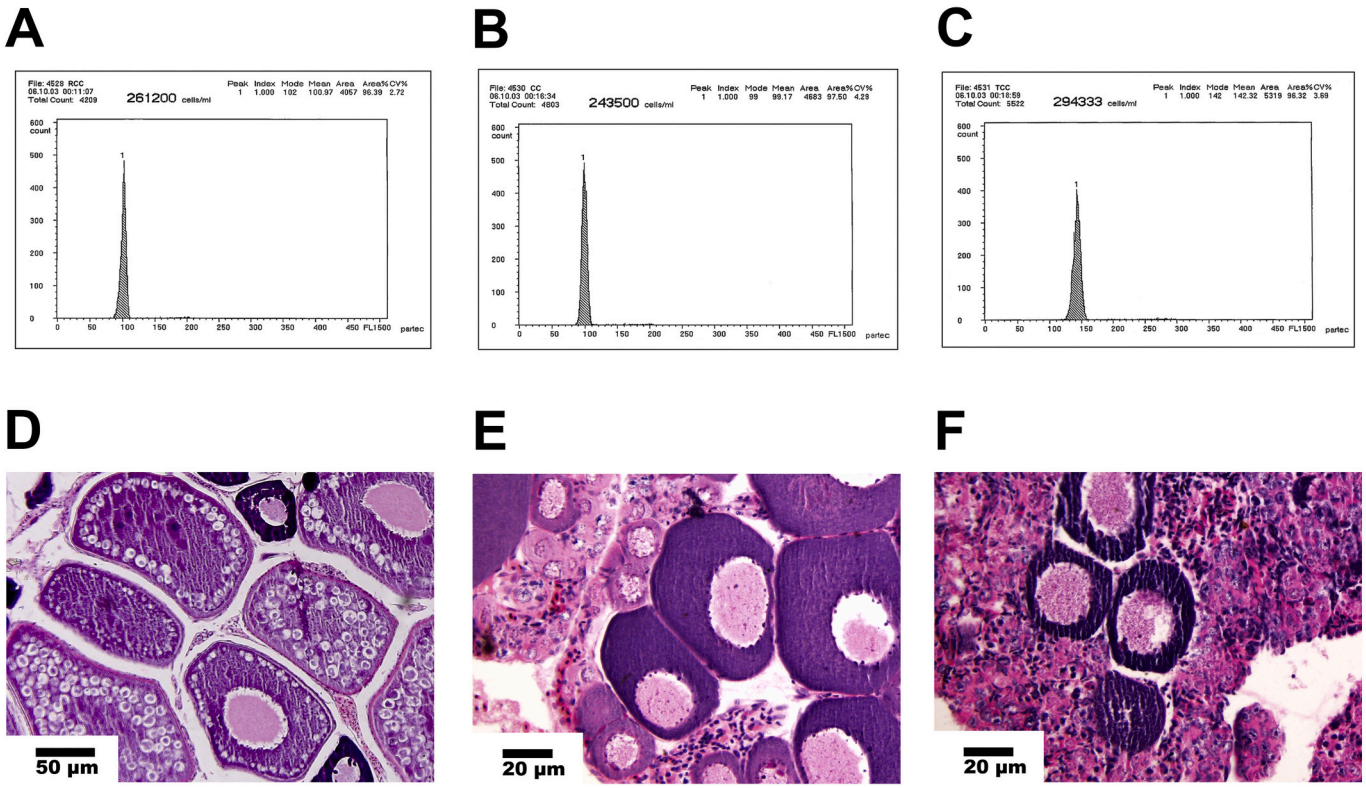

Fig. 1. The DNA content and ovary histological section among RCC, CC, and TCC. The DNA content among RCC (A), CC (B), and TCC (C). The ovary histological section among RCC (D), CC (E), and TCC (F).

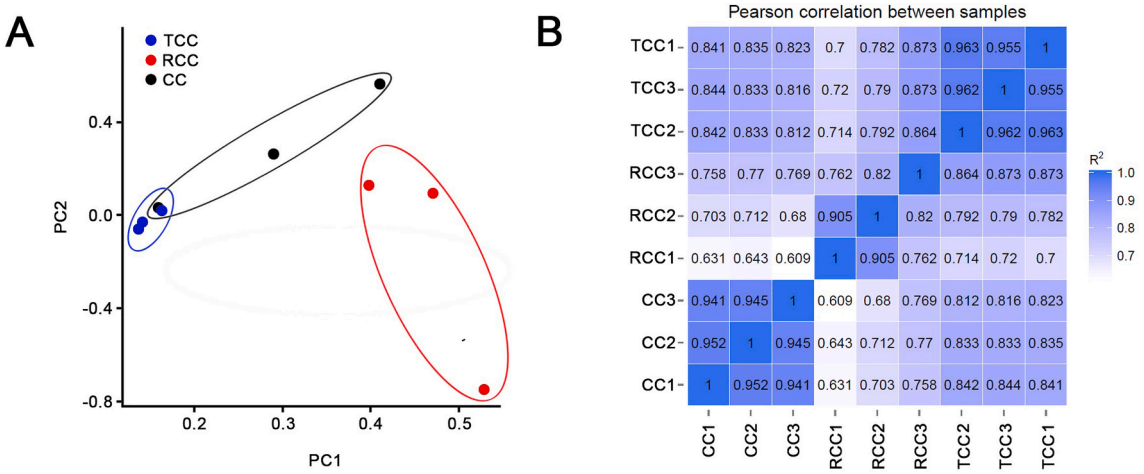

Fig. 2. Diversity analysis among RCC, CC, and TCC. (A) PCA estimation among the triplicate RCC, CC, and TCC. (B) Pearson correlation coefficients among the triplicate RCC, CC, and TCC. The samples were obviously correlated with each other.

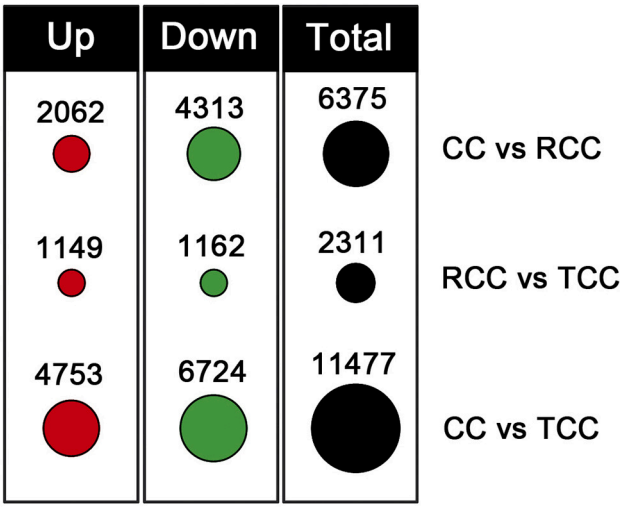

Fig. 3. The bubble diagram of DEGs among RCC, CC, and TCC.

Table 1
Mean DNA content of RCC, CC, and TCC.

Fish type	Mean DNA content[a]	Deviation	Variation coefficient (%)	Ratio	
				Observed ratio	Expected ratio
RCC1	100.97	1.960	2.72		
RCC2	98.80	−0.210	2.78		
RCC3	97.26	−1.750	2.31		
CC1	99.17	1.047	4.29	CC1/RCC1 = 1.02[b]	1
CC2	98.46	0.337	3.30		
CC3	96.74	−1.383	2.84		
TCC1	142.32	−0.583	3.69	TCC1/(RCC1 + 0.5 CC1) = 0.95[b]	1
TCC2	143.53	0.627	3.31		
TCC3	142.86	−0.043	4.03		

differentially expressed in the hypothalamus of TCC and its original parents RCC and CC, 15 representative DEGs (cytochrome P450 family 11 subfamily C member 1 (*cyp11c1*), Estrogen receptor 2b (*esr2b*), keratin 18b (*krt18b*), napsin A aspartic peptidase (*napsa*), protein kinase, cAMP-dependent, catalytic, beta a (*prkacba*), v-akt murine thymoma viral oncogene homolog 2 (*akt2*), v-crk avian sarcoma virus CT10 oncogene homolog (*crk*), fibroblast growth factor 16 (*fgf16*), insulin-like growth factor 1b receptor (*igf1rb*), transforming growth factor beta 2 (*tgfb2*), ATPase H^+ transporting V1 subunit B2 (*atp6v1b2*), lysosomal associated membrane protein 1a (*lamp1a*), Neuronal nitric oxide synthase 1 (*nos1*), T cell immune regulator 1 (*tcirg1a*), thrombospondin 1a (*thbs1a*)) were selected at random and validated by qPCR. The same trends in the expression levels of these genes were detected by qPCR as obtained from RNA-seq data analysis (Fig. 6). These results demonstrated the reliability of RNA-seq data used to analyze differential mRNA expression.

4. Discussion

Hybridization, which has been widely used in aquaculture around the world since the 1980s, is an effective way to prevent variety degradation and produce an improved variety [2]. In our previous study, TCC was demonstrated to have several enhanced performances such as sterility, faster growth rate, stronger disease resistance and stress resistance, and high-quality meat [35]. However, the molecular mechanisms underlying sterility, growth, and disease resistance are poorly understood in triploid fish. In the current study, we constructed RNA-seq libraries of the hypothalamus from RCC, CC, and TCC, and detected some DEGs and pathways that were associated with sterility, growth, and disease resistance of TCC. To the best of our knowledge, this study is the first global transcript analysis of the hypothalamus from triploid fish.

The hypothalamus is a complex area in the brain that participates in homeostasis, including but not limited to energy balance. The hypothalamus-pituitary-gonad axis plays an essential role in reproduction and steroid hormone production in both females and males. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) to stimulate the synthesis and release of pituitary gonadotropins. Gonadotropins act on the gonads to stimulate the production and secretion of gonadal steroid hormones, and ultimately promote gonadal development and maturation, and gametogenesis. The hypothalamus-pituitary growth axis plays a vital role in growth and development in animals. The hypothalamus regulates the secretion of growth hormone by releasing growth hormone-releasing hormone and somatostatin. The growth hormone further binds to the target gland and initiates the signal transduction mechanism, especially the activation of the insulin-like growth factor gene, which finally acts on the receptor of target organs such as muscle, regulates the activity of the target organ.

Steroid hormones play crucial roles in regulating a wide range of physiological processes (including cell growth, differentiation, development, and reproduction) and in overall homeostasis and health, throughout the life of vertebrates [60]. Interfering with steroid biosynthesis may lead to reproductive dysfunction, alterations in sex differentiation, growth, and development as well as the development of

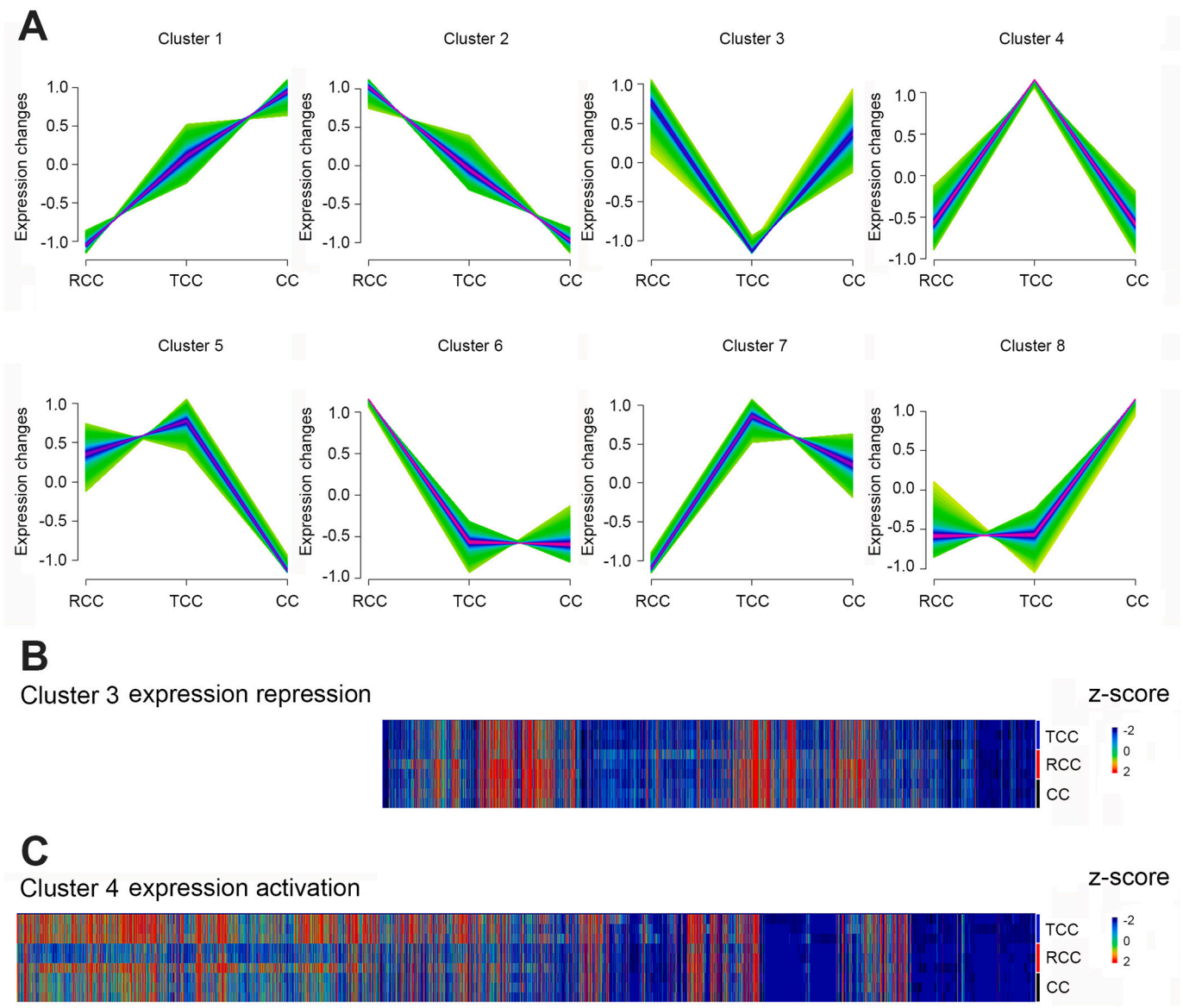

Fig. 4. Fuzzy Cluster Analysis of DEGs. (A) Fuzzy Cluster identified eight distinct temporal patterns of mRNAs expression. The x-axis represented three kinds of cyprinid fishes, while the y-axis represented log2-transformed, normalized intensity ratios in each fish. (B) Hierarchical clustering of 3849 DEGs in Cluster 3 expression repression. (C) Hierarchical clustering of 6005 DEGs in Cluster 4 expression activation.

certain cancers [61]. Steroid hormone synthesis is controlled by the activity of several highly substrate-selective cytochrome P450 enzymes (CYPs) and several steroid dehydrogenases and reductases [61]. In previous studies, the cytochrome P450 family 17 subfamily A member 1 (*cyp17a1*) mutation showed the abnormal development of the genitalia in humans [62]. In zebrafish, *cyp11c1* homozygous mutants had smaller genital papilla and could not mate naturally, and the females showed a reduction in egg spawning and a failure of in vitro germinal vesicle breakdown [63]. Estrogens, one of the sex steroid hormones, serve important functions in sexual differentiation and reproduction, especially in the development and expression of female sexual characteristics [60]. These effects are mostly mediated by estrogen receptors belonging to the nuclear receptor superfamily. *esr2b*, one of the estrogen receptors, is ubiquitous for estrogen-mediated effects and participates in the gonadal development and reproduction in most teleost fish. In previous studies, the single *esr2a* or *esr2b* mutant displayed normal reproductive development and function in both female and male zebrafish. However, double knockout of *esr2a* and *esr2b* caused folliculogenesis arrest at the previtellogenic II stage followed by sex reversal from female to male [64]. In both female and male Nile tilapia, the *esr2b* homozygous mutants displayed completely infertile due to reproductive tract malformation [65]. Matrix metallopeptidases (MMPs) are a special class of proteolytic enzyme that play key roles in follicular development and luteinization in mammals [66]. *mmp2*, one of the key genes of the GnRH signaling pathway and the Estrogen signaling pathway, plays a crucial role in extracellular matrix remodeling during ovarian follicular development, ovulation, and atresia. In previous studies, Imai et al. [67] found that the expression of *mmp2* was positively correlated with the concentration of estradiol and negatively correlated with the concentration of progesterone in 2–6 mm diameter of bovine follicles. mRNA and protein expressions of mmp2 in mouse follicles were proportional to the diameter of the follicles [68]. Tissue sections stained with HE showed that RCC ovaries were contained oocytes in stages II, III, and IV (Fig. 1D), CC ovaries were contained many oocytes in stage II (Fig. 1E), while TCC ovaries were contained only a few oocytes in stage II (Fig. 1F). It is well known that females of RCC reach sexual maturity at one year old, while the females of CC reach sexual maturity at two years old. The above results indicated that the sexual maturity of CC ovaries

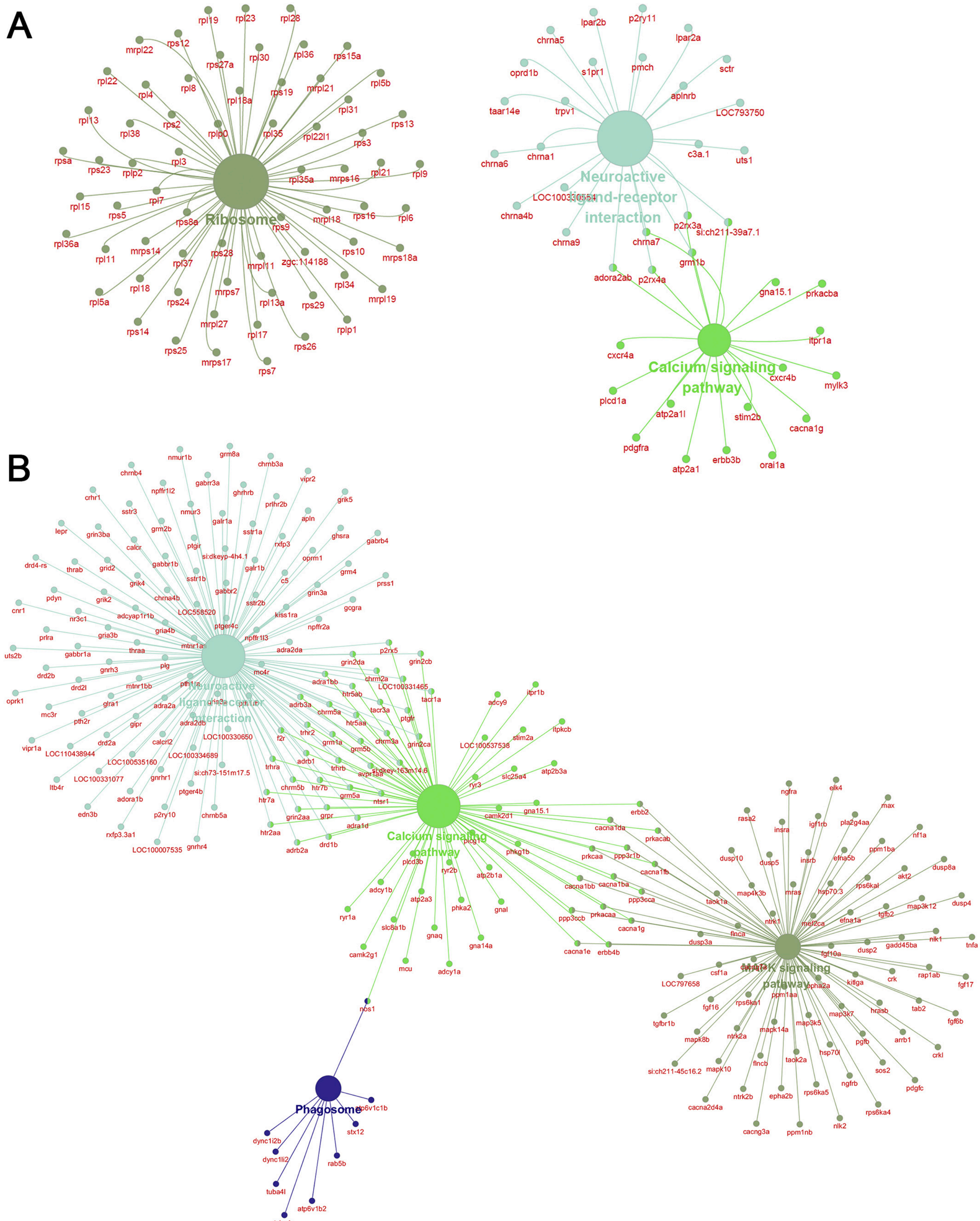

Fig. 5. Protein-protein Interaction Network among RCC, CC, and TCC. (A) Underdominance pattern in TCC. (B) Overdominance pattern in TCC.

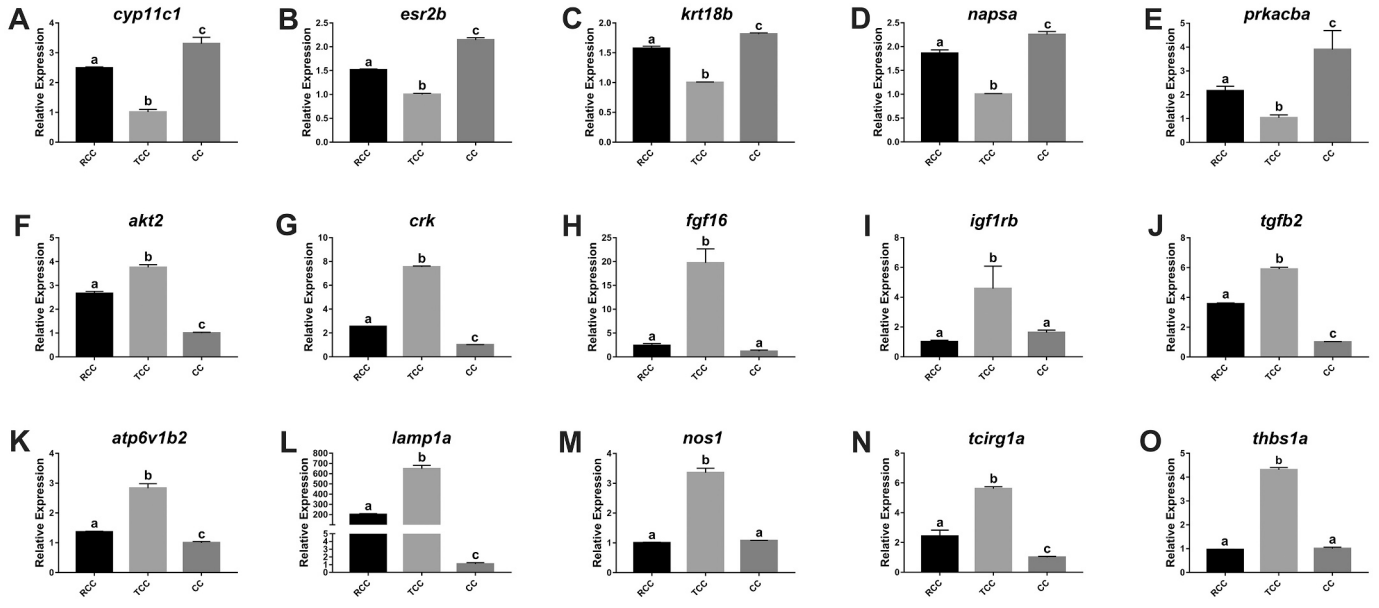

Fig. 6. qPCR verification for 15 DEGs in hypothalamus of TCC and its original parent RCC and CC. (A) cytochrome P450 family 11 subfamily C member 1 (*cyp11c1*). (B) Estrogen receptor 2b (*esr2b*). (C) keratin 18b (*krt18b*). (D) napsin A aspartic peptidase (*napsa*). (E) protein kinase, cAMP-dependent, catalytic, beta a (*prkacba*). (F) v-akt murine thymoma viral oncogene homolog 2 (*akt2*). (G) v-crk avian sarcoma virus CT10 oncogene homolog (*crk*). (H) fibroblast growth factor 16 (*fgf16*). (I) insulin-like growth factor 1b receptor (*igf1rb*). (J) transforming growth factor beta 2 (*tgfb2*). (K) ATPase H^+ transporting V1 subunit B2 (*atp6v1b2*). (L) lysosomal associated membrane protein 1a (*lamp1a*). (M) Neuronal nitric oxide synthase 1 (*nos1*). (N) T cell immune regulator 1 (*tcirg1a*). (O) thrombospondin 1a (*thbs1a*). The different lowercase letters on each bar indicated significant differents ($P < 0.05$).

was ahead of time and RCC ovaries were already mature. Besides, qPCR results demonstrated that all the tested genes were expressed at the highest level in the hypothalamus of CC and the lowest level in that of TCC (Fig. 6A–E). Combined with ovarian tissue sections, these genes were more highly expressed during ovarian maturation. Therefore, we speculated that the reduced expression of these genes may affect the aberrant ovarian development in TCC and exert a role in the sterility of female TCC.

The MAPK signaling pathway plays a crucial role in the regulation of various cellular processes including growth, proliferation, differentiation, stress response, motility, survival, and death [69–71]. The insulin-like growth factor type 1 receptor (IGF1R)-mediated signaling plays a crucial role in growth, development, and physiology. Homozygous *igf1r* null mutant mice exhibited severe growth deficiency (45% of wild-type littermates) and neonatal lethality [72,73]. In zebrafish, knockdown of the *igf1rb* caused embryos that were more growth retarded and developmentally delayed than control MO-injected embryos [74]. The growth and differentiation factor *tgfb2*, one of the members of the TGFBs family, is considered to play crucial roles in multiple developmental processes. *tgfb2* null mutant mice showed perinatal mortality and various developmental defects for single gene disruption [75]. The platelet-derived growth factor (PDGF) signal transduction pathway plays a crucial role in normal development. Deletion of *pdgfc* caused perinatal lethality in homozygous knockout animals, though live births were observed in *pdgfc* knockout mice [76]. A homozygous *pdgfc* mutant mouse showed that most of the growth factor domain was indeed deleted and the expression of the CUB domain was sufficient for viability [77]. The Serine/threonine kinase Akt/PKB plays a crucial role in regulating cell growth, survival, and metabolism. In previous studies, *Akt2/PKBβ*-null mice showed mild growth deficiency [78]. The *akt2*-null zebrafish exhibited partial lethality and severe growth deficiency [79]. The Crk adaptor proteins (Crk and CrkL) play central roles in many biological processes, including growth regulation, cell proliferation, cell adhesion, and migration [80]. *Crk*-null mice died perinatally due to defects in cardiac and craniofacial development [81]. Mice homozygous with a null mutation *CrkL* showed that neural crest-derived cells failed to differentiate and migrate, resulting in defects in cranial and cardiac development [82]. Fibroblast growth factors (FGF) play a role in many developmental and physiological processes including cellular proliferation, migration, differentiation, and homeostasis. Inappropriate expression of FGF is related to various pathological conditions, unregulated cell growth, and tumorigenesis [83]. In previous studies, *fgf6*$^{-/-}$ mutant mice exhibited a severe regeneration defect, accompanied by fibrosis and myotube degeneration [84]. The knockdown of *fgf16* function led to no fin bud outgrowth [85]. In our study, *igf1rb*, *tgfb2*, *pdgfc*, *akt2*, *crk*, *crkl*, *fgf6*, and *fgf16* were expressed at the highest level in the hypothalamus of TCC than in that of RCC and CC (Fig. 6F–J). Together, the large number of genes in the MAPK signaling pathway that were differentially expressed in the hypothalamus of TCC compared with the hypothalamus of RCC and CC suggested the possibility that abnormalities in MAPK biosynthesis may contribute to enhance growth and accelerated body development.

Phagocytes play a central role in fish immunity against infection. The basic function of phagocytes is to identify pathogens and limit the spread and growth of infected pathogens. Phagocytosis refers to the process by which phagocytes transport invading pathogens (such as bacteria and viruses) into cells and remove them. This process constitutes the first line of defense for the innate immunity of organisms. Dysregulation of phagocytosis can cause changes in the immune response and may contribute to autoimmunity. Autoimmunity refers to the rejection of the organism's immune system against its tissues and is a protective mechanism to maintain the stability of the organism. The Phagosome participated in the innate and adaptive immune responses [86,87]. *nos1* is a protein-coding gene that catalyzes the L-arginine to nitric oxide, which is considered as a potent host defense effector in the immune system with antibacterial activity [88]. In previous studies, *nos1* mutant mice led to reduced cytokine production, lung injury, and mortality during the challenge, and *nos1* mutant macrophages had a significant defect in proinflammatory during the bacterial challenge [89]. NOS1-derived nitric oxide promoted macrophages uptake of low-density lipoprotein, thereby inhibiting the progression of atherosclerosis [90]. V-ATPase is ubiquitously expressed and present in endomembrane

organelles such as vacuoles, lysosomes, and endosomes. Malfunction of V-ATPases is associated with multiple diseases, including osteoporosis, deafness, cancer, neurodegeneration, cutis laxa, and renal disease [91,92]. The *atp6v1b2* is highly expressed in the organ of the cerebrum and the organelle of the lysosome [93]. In previous studies, *atp6v1b2* knockdown zebrafish showed developmental defects in multiple organs and systems [92]. In mouse, a heterozygous nonsense *atp6v1b2* mutation impaired lysosomal acidification and resulted in dominant deafness-onychodystrophy syndrome [93]. In humans, a recurrent de novo missense change in *atp6v1b2* cause Zimmermann-Laband syndrome [94]. In addition, a rare gross deletion in *tcirg1a* in Iranian patient led to infantile malignant osteopetrosis [95]. In our study, the *nos1*, *atp6v1b2*, and *tcirg1a* were expressed at the highest level in the hypothalamus of TCC than in that of RCC and CC (Fig. 6K,M,N). Thus, we hypothesized that the elevated expression of these genes may contribute to enhance the disease resistance to pathogen invasion in TCC. This suggested that TCC might possess “improved” innate immune mechanism compared with its original parents RCC and CC.

We evaluated the global gene expression patterns in the hypothalamus among RCC, CC, and TCC. Functional analysis of DEGs showed that the molecular mechanism of female sterility in TCC was closely related to the disruptions of gene expression, and the molecular mechanisms of fast growth and strong disease resistance in TCC were strongly connected with the elevations of gene expression. Our results provide a reference for further breeding sterility, fast-growth, and disease-resistant varieties by distant hybridization. Further physiological and biochemical studies of these candidate genes should be carried out in the future.

Data availability

The transcriptomes data have been submitted to NCBI SRA under the accession numbers PRJNA649388, PRJNA649397, and PRJNA649440.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.01.004.

Author contributions

Shengnan Li: Validation, Data curation, Visualization, Writing - original draft. **Yi Zhou:** Data curation, Visualization, Writing - review & editing. **Conghui Yang:** Data curation, Writing - review & editing. **Siyu Fan:** Investigation. **Lu Huang:** Investigation. **Tian Zhou:** Investigation. **Qiubei Wang:** Investigation. **Rurong Zhao:** Investigation. **Chenchen Tang:** Investigation. **Min Tao:** Conceptualization, Supervision, Validation, Writing - review & editing. **Shaojun Liu:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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