Comparative Transcriptomic Analysis of Gonad Development and Renewal of Ovoviviparous Black Rockfish (Sebastes Schlegelii)

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Abstract

Background: Black rockfish (*Sebastes schlegelii*) has an ovoviviparous reproductive pattern and long-term sperm storage, which resulting in asynchronous gonadal development between the sexes. While the comprehensive understanding of gonad development of black rockfish has not been well studied. Here, we study the gonad development and germ cell renewal by histology and RNA-seq.

Results: In this study, RNA-seq was performed on both testis and ovary to characterize key pathways and genes during development and gamete maturation in black rockfish. Different expression genes (DEGs) were identified and annotated in 4 comparisons (F_III vs F_IV, F_IV vs F_V, M_III vs M_IV and M_IV vs M_V). Based on enriched analysis of DEGs in testis, 11 and 14 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were mapped in M_III vs M_IV group and M_IV vs M_V group, respectively. And DEGs in ovary development periods were also classified into 10 biological function group. The results of the q-PCR expression pattern of the selected genes was significantly correlated with the RNA-Seq results, implying the reliability and accuracy of the RNA-Seq analysis.

Conclusion: The categories intercellular interaction and cytoskeleton, molecule amplification and repairment in cell cycle were revealed to be crucial in testis development and spermatogenesis along with a series of metabolite biosynthesis. Our results provided a comprehensive insight into the black rockfish gonad development for further study of reproductive physiology and molecular biology in ovoviviparity.

Background

Spermatozoa and oocyte in fish species are highly specialized cells fusing into an embryo which will develop into a mature organism producing gametes again[1]. Germ cell development and renewal are required by gonad development during the entire reproductive lifespan of teleost. Spermatogenesis and oogenesis are both highly organized processes and crucial for transmitting the genetic information to next generation[2, 3]. Many aspects of gametogenesis unfold their difference between female and male despite the development of spermatozoa and oocyte follow common principles[2]. Teleost represent the most diverse and numerous group in vertebrates and the researches on spermatogenesis in teleost are mainly focused on a few species such as Atlantic cod (*Gadus morhua*)[4], tilapia (*Oreochromis niloticus*)[5], rainbow trout (*Oncorhynchus mykiss*)[6, 7], zebrafish (*Danio rerio*)[8] and so on. The molecular mechanism of fish spermatogenesis has been reviewed as well[9]. Besides, the studies of oogenesis in teleost fish have been reported in recent decades, including the related gene in tilapia[5], rainbow trout[10], Least killifish (*Heterandria formosa*)[11] and pejerrey (*Odontesthes bonariensis*)[12].

In recent years, some molecular techniques and tools are rising their importance in identification of key pathways and genes in some biological process. RNA-seq platform based on the next-generation sequencing technology (NGS) is considered to be a revolutionary and efficient tool for investigating the key pathways and genes in gonad development. It has been already applied in study of reproduction system in many species including pig (*Susscrofa domestica*)[13], American alligator (*Alligator*

mississippiensis)[14], swimming crab (*Portunus trituberculatus*)[15]. A lot of researches also focused on gonad development and germ cell renewal in teleost revealed the potential mechanism in germ cell and gonad development[16, 17], sex determination and differentiation[18, 19], sex-related genes in both sexes[20, 21].

Fishes take different reproductive strategies including oviparity which is common in most teleost for laying eggs before fertilization, viviparity in some Chondrichthyes species whose embryos develop inside ovary with direct nutrition connect with maternal[22], and the recent well-studied ovoviviparity which eggs fertilized in ovary with development of lecithotrophic larva in some of teleost including the black rockfish (*Sebastes schlegelii*)[23–25] in the present study. Previous studies on black rockfish were mainly focused on environmental toxicology and immune[26–28], response to stress [24, 29], whole genomic data analysis[30, 31] and reproductive physiology[32–35]. While the reproductive studies of black rockfish were most on single gene identification analysis, [32–34, 36, 37] and function[38, 39]. While little is known about the comprehensive understanding of gonad development of black rockfish. In this study, RNA-seq was performed on both testis and ovary to characterize key pathways and genes during development and gamete maturation in black rockfish. The transcripts were de novo assembled and annotated greatly enriched the black rockfish gene database. The identified pathways and genes in this study also provided a novel insight into the reproductive biology of ovoviviparious teleost.

Results

1. Basic physiological data and histology analysis identified different stages of ovary and testis

H&E stain was processed to identified the different stages of gonad development from October to March of the next year. As shown in Fig. 1, testis was characteristic as stage III which spermatogenesis was not observed yet in October until early November when spermatogenesis was completed as a mark for stage IV. After mating in January of the next year, sperm was totally emptied from testis, showed the testis was in stage V. Stage III oocyte was observed in early November with lipid drop and yolk accumulation initiate. Lipid drop and yolk were showed to fuse together in oocyte at stage IV. Completion of the first meiotic division and first polar body discharge were observed at stage V oocyte.

According to the identification by H & E stain, 3 different stages of both gonads were distinguished and collected and named as F_III, F_IV and F_V for stage III, IV and stage V ovary, while M_III, M_IV and M_V for stage III, IV and stage V testis. Average bodyweight and gonad weight were showed in **Additional file 1.** In testis, both high body weight (795.09g ± 58.63 g) and gonadosomatic index (GSI) (1.26) were conventionally observed in winter which mating behavior occurs in ovoviviparous black rockfish. While high body weight (880.68g ± 99.48 g) and GSI (7.57) of ovary were showed in middle March of the next year when the oocytes mature and fertilization was coming.

2. de novo assembly and annotation of black rockfish gonad transcripts

RNA-Seq was performed on both ovary and testis samples from 3 different stages. A total of 1,029,619,820 raw reads (150 bp) were obtained from 18 gonad samples on the Illumina HiSeq X ten platform. After preprocessing and filtration of low-quality sequences, clean read count was 998,950,272 (**Additional file 2**). The *de novo* assembled transcriptome included 517,848 genes with the N50 of 1,660 indicated a high-quality assembly. The genes of black rockfish gonads were annotated in 7 public databases including Nr, Nt, KO, SwissProt, PFAM, gene ontology (GO)and KOG with 61.63% of genes annotated in at least 1 database. The Nonredundant (NR) annotation showed that 72.6% of genes were annotated in 5 fish species with the highest sequence similarity compared to large yellow croaker (*Larimichthys crocea*) (Fig. 2a).

The functional classification of transcriptome data is the basic requirement for the application of functional genomic approaches in fishery research. Analysis based on GO and KEGG database are the common methods in functional classification of transcriptomic sequence. Result showed Blast2Go assigned 123,181 genes into 56 functional GO terms (Fig. 2b). Regarding the 3 primary ontology categories, Biological Process (BP) represents the majority (26 terms) of annotations, followed by Cell Component (CC) (20 terms) and Molecular Function (MF) (10 terms). Based on the analysis of level 2 GO terms, cellular process (GO:0009987) showed the most annotations genes in BP. And for CC, cell (GO:0005623) and cell part (GO:0044464) contained the highest numbers of annotations. The GO terms related to MF with the highest number of annotations were binding (GO:0005488), catalytic activity (GO:0003824). KEGG analysis was performed to understand the higher order functional information of biological system[49]. Based on the analysis, totally 70,174 genes were annotated into 5 categories on 32 significantly enriched KEGG pathways (Fig. 2c).

3. Analysis different expression genes in gonad development stage

A total of 33,393 DEGs were obtained from 4 different gonad development libraries (adjusted p-value < 0.01 and absolute $\log_2$foldchange > 2) (Fig. 3). Among them, 464 DEGs (151 up-regulated and 313 down-regulated) were significantly expressed in F_III vs F_IV, and 329 DEGs (109 up-regulated and 220 down-regulated) were significantly expressed in F_IV vs F_V. While male showed less time in reproductive period revealed much more DEGs than female, which 3,858 DEGs (1,611 up-regulated and 2,247 down-regulated) were significantly expressed in M_III vs M_IV and 30,160 DEGs (24,446 up-regulated and 5,714 down-regulated) were significantly expressed in M_IV vs M_V.

3.1 Identification of DEGs in testis development of black rockfish

Based on these DEGs mentioned above, 3,858 and 30,160 annotated DEGs were obtained from M_III vs M_IV group and M_IV vs M_V group, respectively (Fig. 4a). Heatmap analysis of these 32,772 DEGs expressed in all 3 stages of testis revealed that most of these genes in both stage III and IV testis showed similar expression pattern, which up-regulated genes in stage III (M_III) and stage IV testis (M_IV) showed down-stream in stage V (M_V) testis, suggested that the expression profile of these DEGs and male reproductive process including gamete mature were directly proportional (Fig. 4b). GO analysis of these

DEGs showed most of them were mapped onto MF term (*p value* < 0.01), especially molecule binding GO terms (anion binding, GO:0043168, small molecule binding, GO:0036094 and so on) (Fig. 4c).

11 and 14 significantly enriched KEGG pathways were mapped between M_III vs M_IV group and M_IV vs M_V group (*p-value* < 0.01), respectively (Table 1).

In M_III vs M_IV group, 11 KEGG pathways were classified into 3 categories, including **intercellular interaction and cytoskeleton**, **molecule amplification and repairment in cell cycle**, and **other** (Fig. 5). The DEGs in category **intercellular interaction and cytoskeleton**, including extracellular matrix (ECM)-receptor interaction, focal adhesion and regulation of actin cytoskeleton, were basically up-regulated in stage IV compared with stage III. DEGs in category **molecule amplification and repairment in cell cycle**, including cell cycle, ubiquitin mediated proteolysis, DNA replication, Fanconi anemia pathway, RNA transport and mRNA surveillance pathway, were significantly enhanced in stage III, when the spermatogenesis start, cell division and protein biosynthesis are processing, compared with stage IV. In addition, some other pathways such as steroid hormone biosynthesis was up-regulated in stage IV testis.

As shown in Fig. 6, 14 KEGG pathways were classified into 5 categories, including **progesterone induced gamete mature**, **molecule amplification and repairment in cell cycle**, **endoplasmic reticulum-related protein processing and exocytosis in nervous system**, **infection and immune related** and other in M_IV vs M_V group. In category **progesterone induced gamete mature**, most DEGs showed up-regulated in stage IV testis due to the importance of steroid hormone to germ cell division. Category **molecule amplification and repairment in cell cycle**, which also showed up-regulated in both stage III and stage IV, implied the drastic changes in cell metabolism continue the whole reproductive process. The protein processing in endoplasmic reticulum, synaptic vesicle cycle and retrograde endocannabinoid signaling in categories **endoplasmic reticulum-related protein processing and exocytosis in nervous system** showed the most interest and abundant DEGs. DEGs, such as *sar1b*, *sec13*, *sec24c* and *slc18a2* are involved in transport vesicles, *stx2* is related to epithelial morphogenesis due to exocytosis, *cacna1b* regulates hormone or neurotransmitter release, while *gria1* and *grm1* for receipting glutamate neurotransmitter message. These results suggested these genes expressing variated significantly between stage III and IV testis were inseparable with the intense reproductive stages.

As shown in **Additional file 3**, 3 KEGG pathways cell cycle, mRNA surveillance pathway and RNA transport, which were both up-regulated in M_III vs M_IV and M_IV vs M_V, indicated that the activity process in the whole reproductive cycle. Interestingly, Ubiquitin mediated proteolysis, which was highly expressed in M_III vs M_IV and M_IV vs M_V, presented totally different DEGs, suggested that the pathway may play different roles in different development stages. In Oocyte meiosis pathway, *sgo1*, *stag3*, *smc1b* and *smc3* were up-regulated in stage IV, suggested the exit of meiosis of gamete in final reproductive stage. Besides, DEGs in p53 signaling pathway, *ccne1*, *ccnd2*, *cdk2* with up-regulated and *srsf10* with down-regulated expressed profile, which *srsf10* repressed cell cycle G1 and G2 phase arrested by inhibition of *ccne1*, *ccnd2*, *cdk2*, resulting cell cycle was active in stage III and IV testis.

Table 1
significant enriched KEGG pathways in M_IIIvsM_IV group and M_IVvsM_V group.

Stage	KEGG pathway	ID	Input gene	Regulation
M_IIIvsM_IV				
Intercellular interaction and cytoskeleton	ECM-receptor interaction	ko04512	39	DOWN
	Focal adhesion	ko04510	64	DOWN
	Regulation of actin cytoskeleton	ko04810	59	DOWN
Molecule amplification and repairment in cell cycle	**Cell cycle**	ko04110	39	UP
	Ubiquitin mediated proteolysis	ko04120	27	UP
	DNA replication	ko03030	10	UP
	Fanconi anemia pathway	ko03460	12	UP
	mRNA surveillance pathway	ko03015	23	UP
	Steroid hormone biosynthesis	ko00140	21	DOWN
	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	ko00563	7	UP
M_IVvsM_V				
Progesterone induced gamete mature	Progesterone-mediated oocyte maturation	ko04914	117	UP
	Oocyte meiosis	ko04114	129	UP
Molecule amplification and repairment in cell cycle	**mRNA surveillance pathway**	ko03015	97	UP
	Cell cycle	ko04110	140	UP
	Ubiquitin mediated proteolysis	ko04120	143	UP
	RNA transport	ko03013	139	UP
	p53 signaling pathway	ko04115	69	UP
	Apoptosis - multiple species	ko04215	34	UP
endoplasmic reticulum-related protein processing and exocytosis in nervous system	Protein processing in endoplasmic reticulum	ko04141	159	UP
	Synaptic vesicle cycle	ko04721	102	UP

Stage	KEGG pathway	ID	Input gene	Regulation
	Retrograde endocannabinoid signaling	ko04723	179	UP
Infection and immune related	Staphylococcus aureus infection	ko05150	49	DOWN
	Transcriptional misregulation in cancer	ko05202	174	DOWN
	Pertussis	ko05133	73	DOWN
	RNA transport	ko03013	30	DOWN
	Huntington's disease	ko05016	205	UP

3.2 Identification of DEGs in ovary development of black rockfish

Due to the special reproductive strategy in female black rockfish, the transcriptomic level changes in ovary seemed not as much drastic as testis. Annotation and enrichment analysis only achieved 464 and 329 DEGs (adjusted p-value < 0.01 and absolute $\log_2$foldchange > 2) in F_III vs F_IV group and F_IV vs F_V group, respectively (Fig. 7a). The expression pattern of all these 765 DEGs were different from testis, which up-regulated in stage IV (F_IV) and V (F_V) ovary, suggested the delayed development in ovary compared with testis (Fig. 7b).

Interestingly, GO analysis of these 765 DEGs with a total of 205 DEGs were annotated in membrane (GO:0016020) in CC (Fig. 7c). **Additional file 4** showed the detail of these 205 DEGs, most of which participated in membrane-anchored enzymatic reaction or molecular transport. 10 accurately biological function classifications and several subclassifications were accomplished with **cell cycle**, **cell junction**, **cell structure**, **metabolism**, **DNA binding and transcription regulated**, **immune system**, **nervous system**, **molecular transport**, **protein modification**, **RNA binding**, and **signal transduction** including. Among these classifications, **Molecular transport** showed the most abundant (40 DEGs) of all, indicated the importance of matter transport process during ovary development stage of black rockfish.

4. Validation of RNA-Seq results by qRT-PCR

10 DEGs were randomly selected for q-PCR analysis to validate the present RNA-Seq data. The results showed that the q-PCR expression pattern of the selected genes was significantly correlated with the RNA-Seq results (R^2: 0.933–0.9559). Totally, the RNA-Seq data were confirmed by the q-PCR results, implying the reliability and accuracy of the RNA-Seq analysis (Fig. 8).

Discussion

The black rockfish, as an economically crucial fish species, has been processed a lot of works, especially in reproductive and toxic physiology and molecular biology due to the unusual ovoviviparous strategy[24, 27, 30]. While the transcriptomics profile of the black rockfish gonad development was less clear. In order to investigate underlying mechanisms, a series of sex-related genes and biological pathways should be determined for further exploration. The present study mainly focused on identification of the key genes and pathways in both ovary and testis development and germ cell renewal by histology and RNA-seq.

Identification and terminology of gonad development of the black rockfish

Reproduction of most teleost is an annual and cyclic event, while gonads present different development stages for germ cells renewal. Critical phases of fish reproductive cycle were determined by a conceptual model based on specific histological and physiological markers[50]. Black rockfish testis go through 4 phases during development, including the developing phase when no spermatozoa present in lumen of lobules or sperm ducts (described as stage III in the present study), spawning phase when spermatogenesis active (described as stage IV), and regressing phase with few or no sperm (described as stage V) [50, 51]. Different from the oviparity strategy, black rockfish ovulated eggs fertilized and retained in ovary during the gestation phase[50]. In the present study, the description of the developmental stage of ovary as stage III, stage IV and stage V ovary indicated the terminology of developing phase, early developing subphase and actively spawning subphase, respectively[50, 52].

Key pathways in testis development and gametogenesis of male black rockfish

Testis of male black rockfish showed drastic variation from stage III to stage V which reflected in both histology results and transcriptomic profile. During the development from stage III to stage IV, one category of pathways **intercellular interaction and cytoskeleton** showed upregulation significantly. Similar to mammals, cellular interactions among sertoli cells and germ cells of fish in the seminiferous epithelium play important structural and functional roles as well. Testis structure undergo dramatic morphofunctional changes during reproductive season to activate spermatogenic arrangement[53, 54]. It suggested that actin-related adhesion among sertoli cells and sertoli cells to germ cells are associated with multiple events crucial for spermatogenesis and normal fertility, especially for spermatid elongation in stage IV testis[54, 55]. In the transcriptomics analysis of spotted knifejaw (*Oplegnathus punctatus*), similar intercellular interaction pathway (cell adhesion molecules) was significantly enriched in testis as well[20].

In fish, cytoplasmic extensions of sertoli cells form a envelop of a single synchronously developing group of germ cells derived from a single spermatogonium. The form of the group of germ cells, namely spermatogenesis is a highly organized and coordinated process in which diploid spermatogonia proliferate and differentiate to mature spermatozoa [2]. Both processes required a large amount of

molecule, including DNA, RNA, protein, lipid and steroid, for cell amplification. In the present study, category **Molecule amplification and repairment in cell cycle**, including cell cycle, ubiquitin mediated proteolysis, DNA replication, fanconi anemia pathway, mRNA surveillance pathway, RNA transport, p53 signaling pathway and apoptosis, showed significant up-regulated in both stage III and IV testis. Different from mammals, fish show a cystic type of spermatogenesis and go through different stages by an accompanying group of sertoli cells. The shape shifting of a cystic which is enveloped by sertoli cells is accompanied by strong proliferation of sertoli cells[56, 57]. In African catfish and Nile tilapia, sertoli cell proliferation occurred primarily during spermatogonial proliferation[57]. In addition, in *Leporinus taeniatus* spermatogenesis is also processed as which diploid spermatogonia proliferate and differentiate to haploid spermatozoa[58]. On the other hand, fanconi anemia pathway was confirmed as an efficient DNA repairment pathway[59, 60]. In fanconi anemia disease causing genes mutant zebrafish, the DNA damage repairment failed and caused a series of abnormal development.

Testis development and spermatogenesis required a mass of metabolites, especially for androgens. In fish, transduction of the gonadotropin signal stimulates the production of 11-ketotestosterone (11-KT), a major androgen for fish [61], functionally activity the androgen receptors[62, 63]. To complete the process, enzymes and other proteins are required for biosynthesis[64]. In the present study, the steroid hormone biosynthesis pathway and the key genes *3b-hsd* and *cyp11a1* were significant up-regulated in stage IV testis, indicating the importance of steroidogenesis for spermatogenesis [62, 65]. A few pathways related to RNA transcription and translation transportation, namely RNA transport, mRNA surveillance pathway and ubiquitin mediated proteolysis, were also significantly enriched in stage IV testis, suggested the metabolism and synthesis under gonadotropin and steroid hormone have been crucial for maintenance of spermatogenesis[62, 66]. Interestingly, these pathways (progesterone-mediated oocyte maturation and oocyte meiosis) were both enriched in stage IV testis, implying the importance of the role that progestogen plays in spermatogenesis. Serum 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), as fish specific progestin, exhibited higher in stage IV concomitant with testicular development in male turbot (*Scophthalmus maximus*)[67]. Knock out of the nuclear receptor of DHP resulted a smaller testis and a lower GSI compared with normal testis in Nile tilapia (*Oreochromis niloticus*)[68].

Key genes in ovary development and gametogenesis of female black rockfish

Due to the special reproductive strategy in female black rockfish, the transcriptomic level changes in ovary seemed not as much drastic as testis. A total of 765 DEGs were enriched significantly which classified into 10 classifications including cell cycle, cell junction, cell structure, metabolism, DNA binding and transcription regulated, immune system, nervous system, molecular transport, protein modification, RNA binding, signal transduction and unclassified genes.

Similar to the testis of black rockfish, ovary development presented significantly enriched DEGs in cell cycle and junction and cytoskeleton as well. The matrix metalloproteinases (MMPs) are a family of

extracellular proteinases which plays a role in the ECM remodeling associated with many physiological and pathological processes[69]. Previous studies in mammal have shown increased MMP19 expression in preovulatory follicles[70], and estrogen receptor knockout lead to down-regulation of *MMP19* and failure in release of mature oocyte[71]. In the present study, *mmp19* was also up-regulated in stage IV ovary, which may be due to follicles mature and rupture afterward in black rockfish.

Black rockfish *vipr* gene showed a significant increase in stage IV ovary implied its critical role in folliculogenesis. Both neuropeptides vasoactive intestinal polypeptide (vip) and pituitary adenylate cyclase-activating polypeptide (pacap) are considered to stimulate the ovarian functions such as steroidogenesis, cAMP accumulation in rat granulosa cells[72] through the PACAP/VIP receptors. VIPR has been found equal affinity with both PACAP and VIP[73]. In zebrafish, the *vipr* expression maintained high during follicle until full-grown stage[74].

The classification **molecular transport** showed the most DEGs in ovary development of black rockfish with 20 genes of solute carrier (SLC) gene superfamily enriched. The SLC gene superfamily encodes series of membrane transporters[75] including passive transporters, cotransporters and exchangers in various cellular membranes[76]. The members of *slc6* gene family (*slc6a11*, *slc6a15*, *slc6a6*, *slc6a8*) which performs Na^+ and Cl^- dependent neurotransmitter transport for γ-aminobutyric acid (GABA), creatine and taurine were differentially expressed along ovary development. The result was coincident with the transcriptomic analysis of hapuku (*Polyprion oxygeneios*)[77]. However, these transport proteins were more commonly mentioned in central nervous system of vertebrates[78], which imply the SLCs may also functional in ovary. Zinc is necessary for meiotic in zebrafish and mammals[79, 80], the zinc transport proteins *slc30* gene family was found expressed in oocyte and cumulus cells during maturation in mouse (*Mus musculus*)[81], which may be a proper explanation that *slc30a9* showed up-regulated in early oocyte mature for zinc transport.

Conclusions

The present study is the first report of the transcriptomic information of the ovoviviparous black rockfish gonad development. Several important candidate pathways and genes in both testis and ovary development have been identified. Among these pathways and genes, the categories **intercellular interaction and cytoskeleton**, **molecule amplification and repairment in cell cycle** were revealed to be crucial in testis development and spermatogenesis along with a series of metabolite biosynthesis. Some key genes emerged in ovary development such as *mmp19* and neuropeptide receptor *vipr* in follicles mature and rupture and the membrane transporter family *slc6* and *slc30* in various ways. These data provided a comprehensive insight into the black rockfish gonad development for further study of reproductive physiology and molecular biology in ovoviviparity.

Materials And Methods

1. Animal and sample collection

Together, 27 adult male and 27 adult female black rockfish cultured in northern Yellow Sea were obtained from October to March of the next year. Nine individuals were sampled for each development stage in all 3 development stages in both sexes. Fish were acclimatized at a density of 10 individuals per tank (diameter 1 m, height 1.5 m) under laboratory conditions for 2 days without feeding. After acclimation, individuals were anesthetized with MS222 (200 mg /L). Body weight and gonad weight were measured and GSI was calculated. Gonad were also collected immediately for both histology and RNA isolation.

2. Histology analysis and RNA isolation

Testis and ovary of different development stages were fixed in Bouin's solution, dehydrated and embedded in paraffin. Tissue sections were cut into 6 μm by a microtome (Leica, Wetzler, Germany) and stained with hematoxylin-eosin. All section photos were taken by Olympus bright field light microscope (Olympus, Tokyo, Japan).

Gonads were collected and frozen in liquid nitrogen for further total RNA isolation with TRIzol reagent (Invitrogen, USA). The quality and concentration of the total RNA were assessed by agarose gel electrophoresis and Agilent 2100 Bioanalyzer system (Agilent Technologies, USA), respectively.

3. Library construct and transcriptome sequencing

In order to mask the difference among sample repetitions, equal amount of total RNA from 3 individual ovaries or testis in same development were pooled together. 18 sequencing libraries were generated NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's instructions and index codes were added to attribute sequences to each sample. Samples were sequenced on an Illumina Hiseq X ten platform and 150 bp paired-end reads were generated. Raw sequences were deposited in the Short Read Archive of the National Center for Biotechnology Information (NCBI) with accession numbers of PRJNA573572.

4. De novo assembly and annotation of sequencing reads

De novo assembly was performed on gonad clean reads using the Trinity assembly software suite[40] without a reference genome. Transcripts (both contigs and singletons) were annotated by BLASTx searches[41] using NCBI non-redundant (Nr), NCBI nucleotide sequences (Nt) and Swiss-Prot databases with a cutoff "e-value" of < $1e^{-5}$. Domain-based comparisons with Protein family (Pfam) and KOG (a eukaryote-specific version of the Clusters of eukaryotic Ortholog Groups) databases were performed by RPS-BLAST tool from locally installed NCBI BLAST + v2.2.28 and HMMER 3.0 program, respectively. Annotated transcripts were analyzed to GO classification with the aid of Blast2Go program[42]. These gene terms were then enriched on the three GO categories (Biological Process, Cellular Component and Molecular Function at level 2) using the GOseq R package[43]. KEGG, which is a database of biological

systems, maps were retrieved by online KEGG Automatic Annotation Server for the overview of metabolic pathway analysis[44, 45].

5. Differential gene expression analysis

The reads of each library were mapped to the *de novo* assembled transcripts with the bowtie 2 program for mismatch check[46]. Count numbers of mapped reads and FPKM (expected number of Fragment Per Kilobase of transcript sequence per Millions base pairs sequenced) were achieved and normalized by RSEM V1.2.15[47]. Differential expression statistical analysis of different development stage of gonad was conducted by the DEGSeq R package[48] with a cutoff "q-value" of 0.01 and $|\log_2(\text{fold change})|>2$. Transcripts with absolute fold change values over 2.0 were marked as significantly differential expressed genes.

6. Experimental validation by quantitative real-time PCR

Expression analysis of 10 selected DEGs were performed by quantitative real-time PCR (qPCR) with specific primers validate our Illumina sequencing data. Primers were listed in **Additional file 5**. Samples were generated from F_III, F_IV, F_V ovary and M_III, M_IV, M_V testis in the preceding experiment. After RNA extraction and reverse transcription, all the cDNA products were diluted to 500 ng/μL. The 20μL qPCR reaction mixture consisted of 2μL cDNA template, 0.4μL of both primers, 10μL of KAPA SYBR®FAST qPCR Master Mix (2X), 0.4μL of ROX and 6.8μL of RNAase-free water. PCR amplification was performed as that incubated in a 96-well optical plate at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s, and a final extension at 72 °C for 2 min. qPCR was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems) and $2^{-\Delta\Delta CT}$ method was used to analysis the expression level of genes.

Abbreviations

DEGs: Different expression genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; NGS: The next-generation sequencing technology; GSI: Gonadosomatic index; GO: Gene ontology; NR: Nonredundant; BP: Biological Process; CC: Cell Component; MF: Molecular Function; ECM: Extracellular matrix; 11-KT: 11-ketotestosterone; DHP: 17α, 20β-dihydroxy-4-pregnen-3-one; MMPs: Matrix metalloproteinases; vip: vasoactive intestinal polypeptide; pacap: pituitary adenylate cyclase-activating polypeptide; SLC: Solute carrier; GABA: γ-aminobutyric acid; NCBI: the National Center for Biotechnology Information; Nr: non-redundant; Nt: nucleotide sequences; Pfam: Protein family; KOG: a eukaryote-specific version of the Clusters of eukaryotic Ortholog Groups; qPCR: quantitative real-time PCR;

Declarations

Ethics approval and consent to participate

All procedures involved in dealing of fish in this study were approved by Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201) prior to the initiate of the study. The studies did not involve endangered or protected species. And all experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in China.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analysed during this study were deposited in the Short Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) of the National Center for Biotechnology Information (NCBI) with accession numbers of PRJNA573572.

And other data supporting the conclusion of this article is included within the article, and can be found in the additional files.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

WHS, LJF and QX designed the study; LJS performed the transcriptome and qRT-PCR experiment; LLK, WXJ, YYJ and LJS performed in samples collection; LJS wrote the manuscript and QX provided manuscript editing and feedback; All authors read and approved the final manuscript.

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Figures

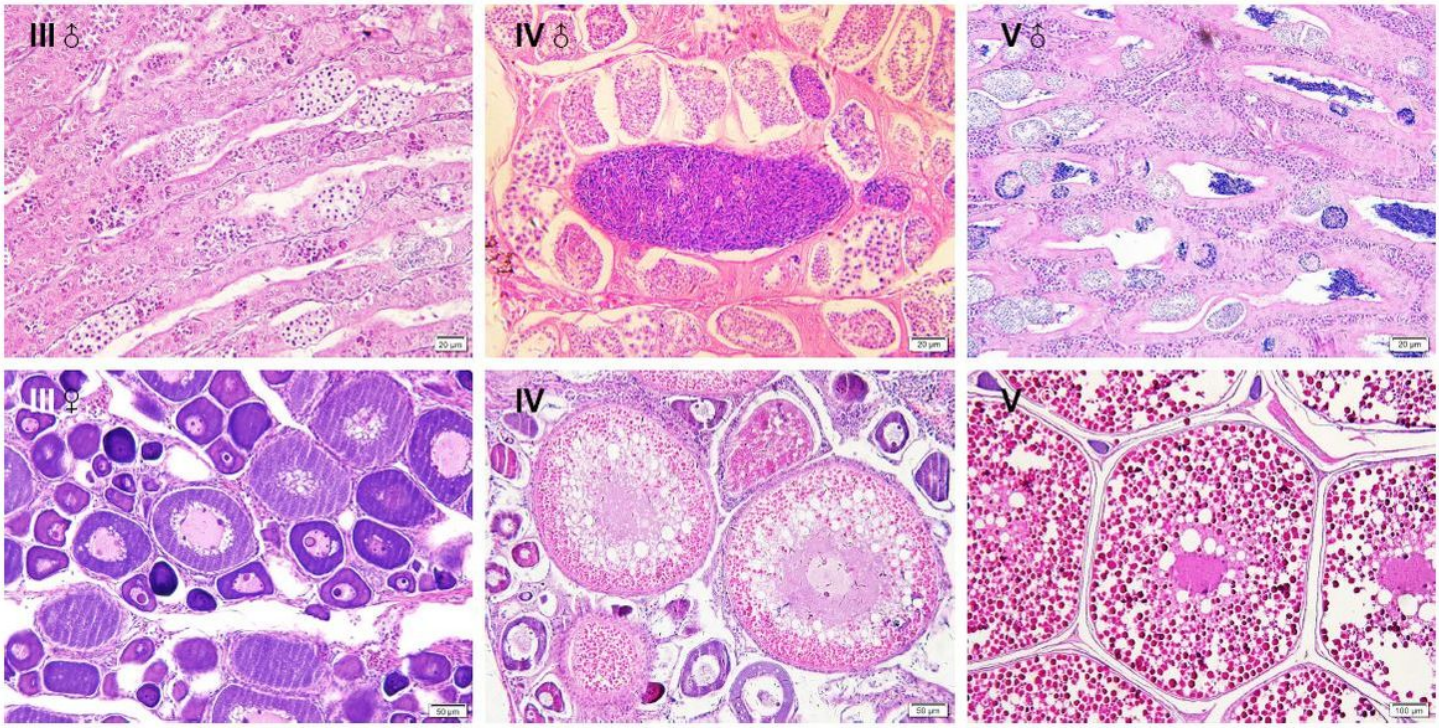

Figure 1

H&E stain of gonad development stage of black rockfish. III♂, IV♂ and V♂represent stage III, IV and V testis, respectively. And III♀, IV♀ and V♀represent stage III, IV and V ovary, respectively.

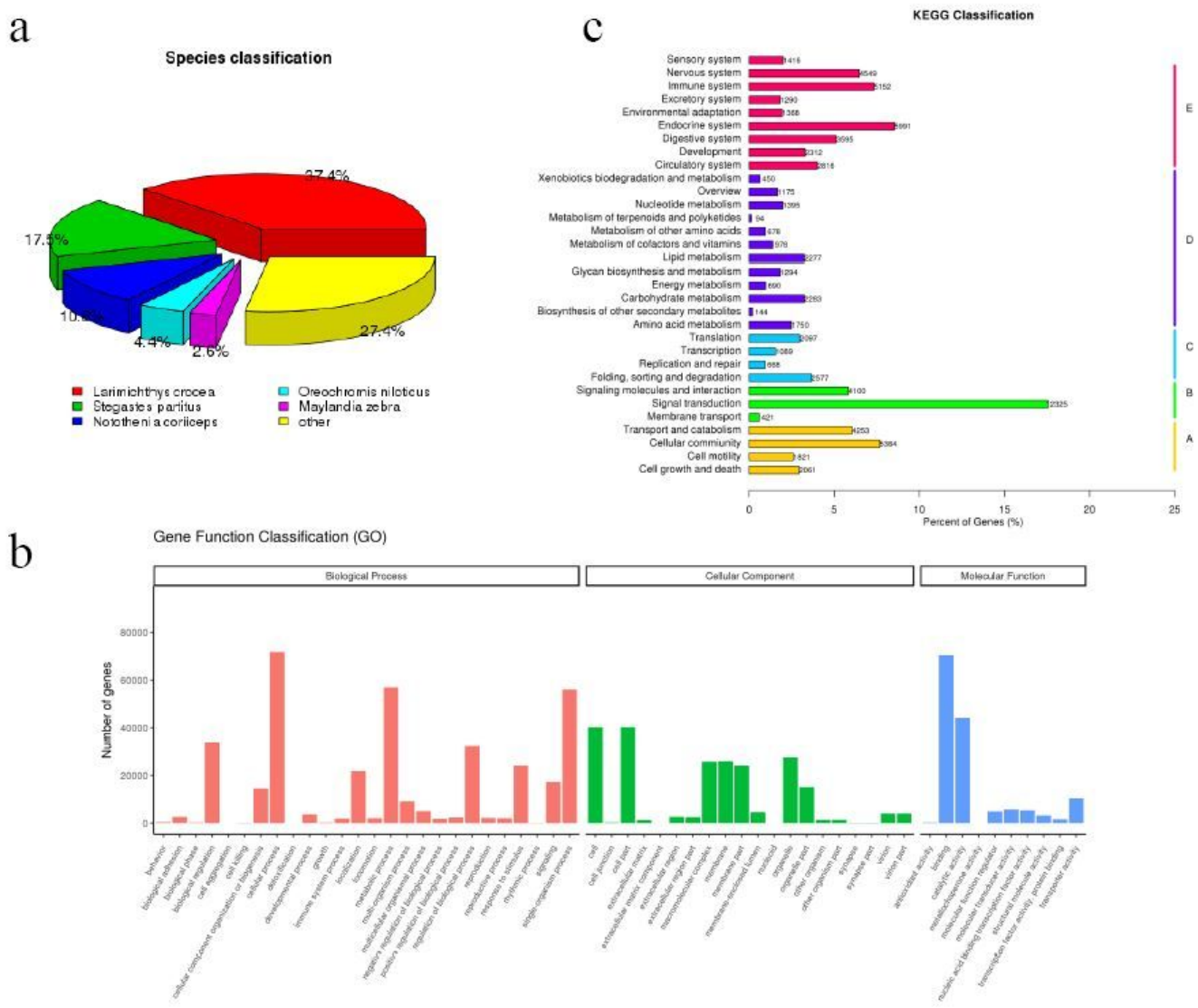

Figure 2

Annotation and functional classification of transcripts in gonad of black rockfish. (a) Top-hit species distribution of BLASTX matches of assembled transcripts. (b) Function annotation of assembled transcripts based on gene ontology (GO) analysis. GO analysis was performed at level 2 for the three main categories (biological process, cellular component and molecular function). The x-axis shows the specific terms. The y-axis shows the number of transcripts in each term. (c) Pathway assignment based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Transcripts were classified into five main categories (A: cellular process, B: environmental information processing, C: genetic information processing, D: metabolism and E: organismal systems).

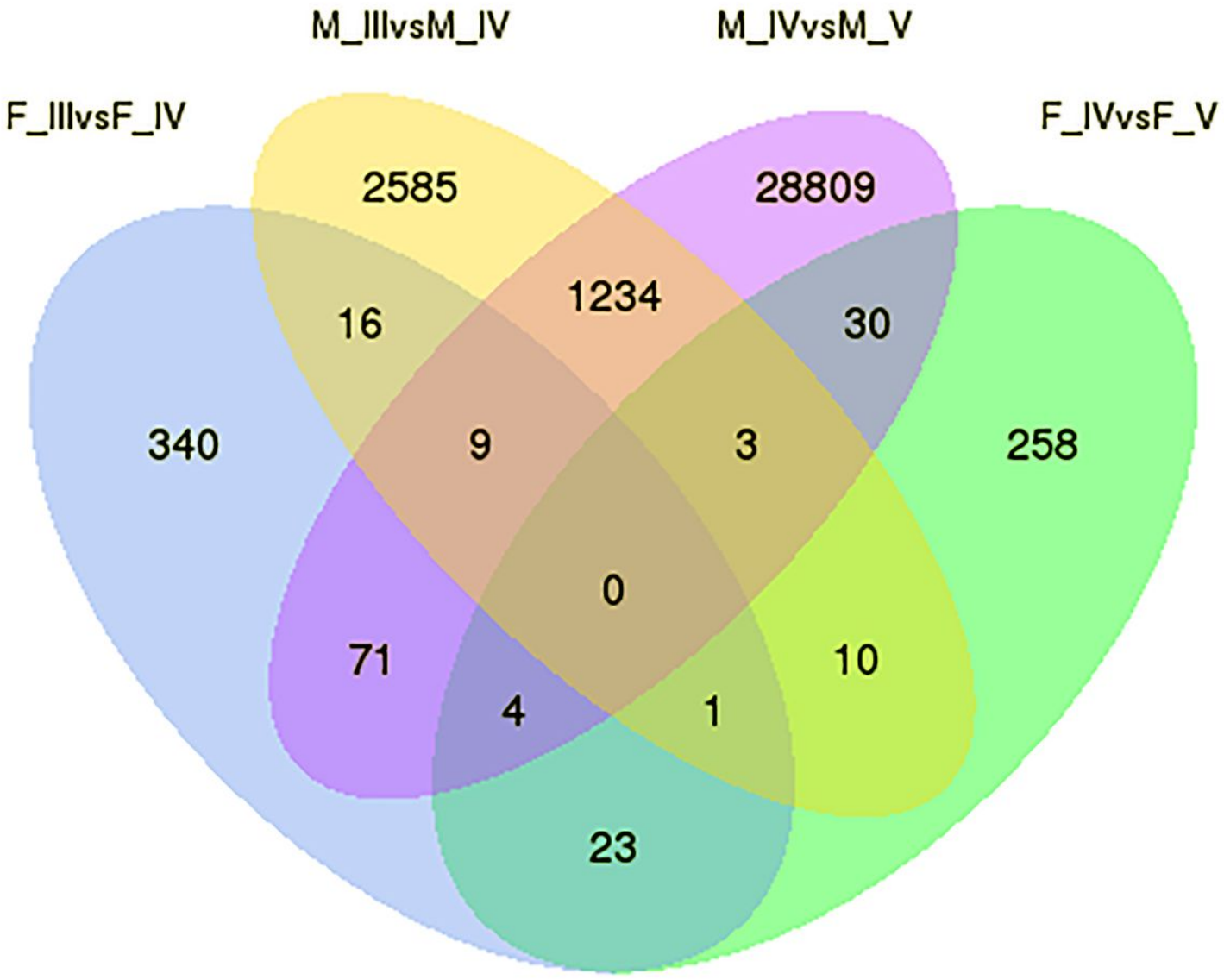

Figure 3

Venn diagram of the DEGs of 4 comparisons in the gonad of black rockfish.

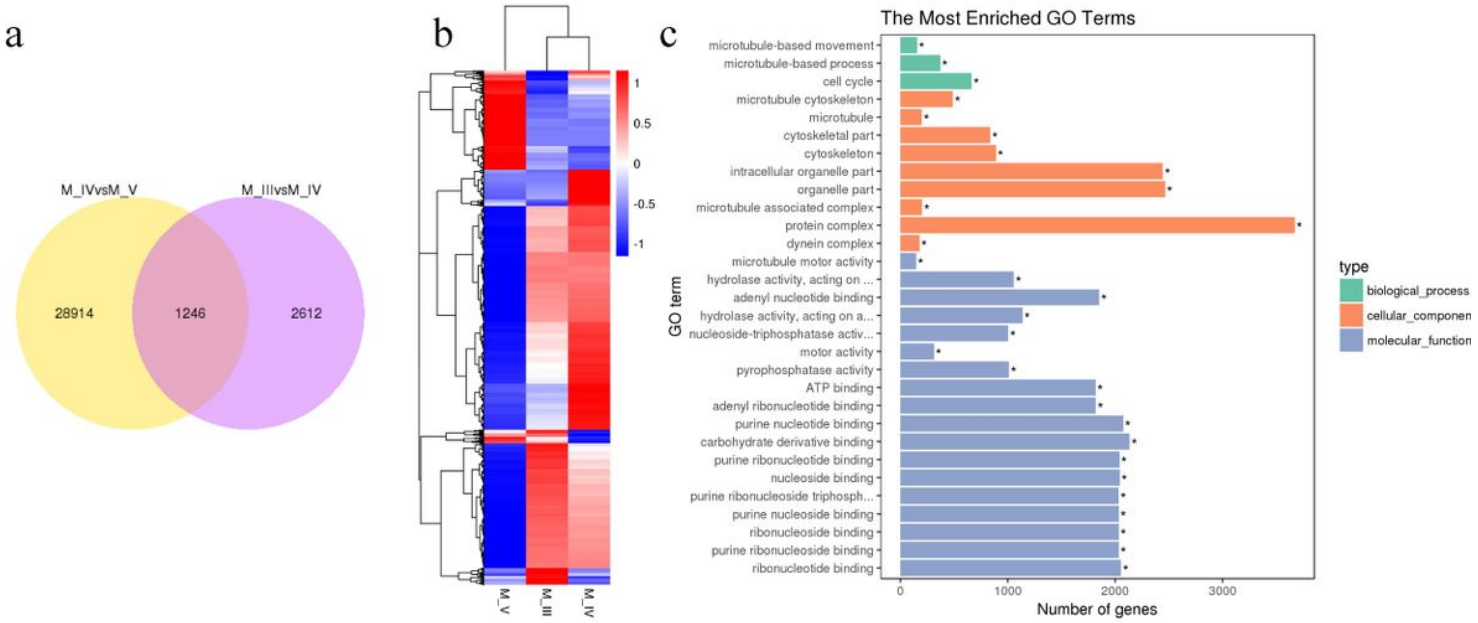

Figure 4

Identification and annotation of DEGs in different development stage in testis of black rockfish. (a) Venn diagram of the DEGs of M_III vs M_IV and M_IV vs M_V. (b) Expression values of 32,772 DEGs in 3 libraries (M_III, M_IV, M_V) are presented in heat map. Red and blue colors indicate up- and down-regulated transcripts, respectively. (c) Gene ontology (GO) analysis for the 32,772 DEGs. The x-axis shows the number of genes in each term. The y-axis shows the specific terms. The asterisk represents the corrected p-value <0.05 in each GO term.

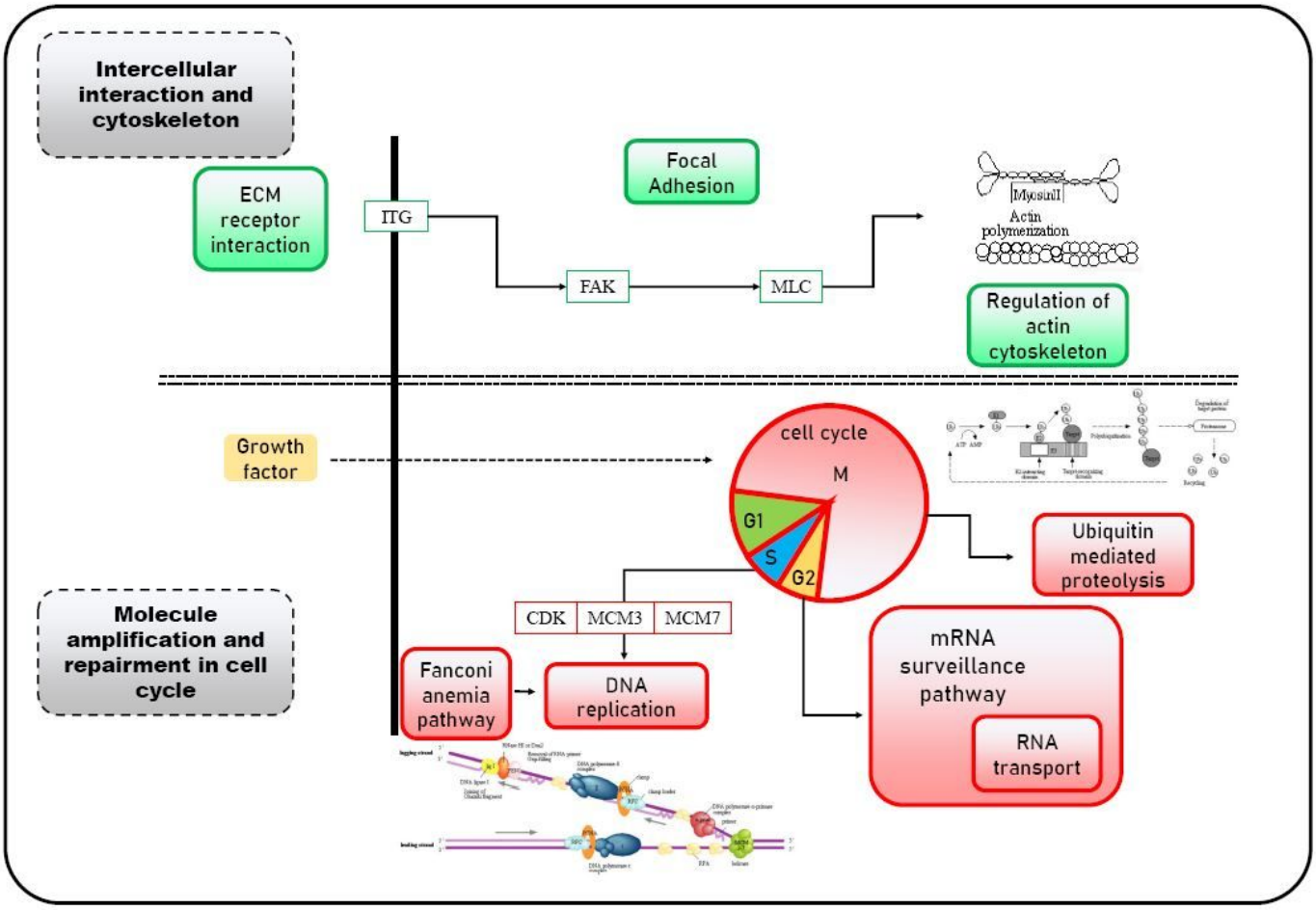

Figure 5

Regulation of pathways in M_III vs M_IV group of black rockfish. Red and green orthogon represent up and down regulated KEGG pathways and DEGs, respectively(45, 49, 82).

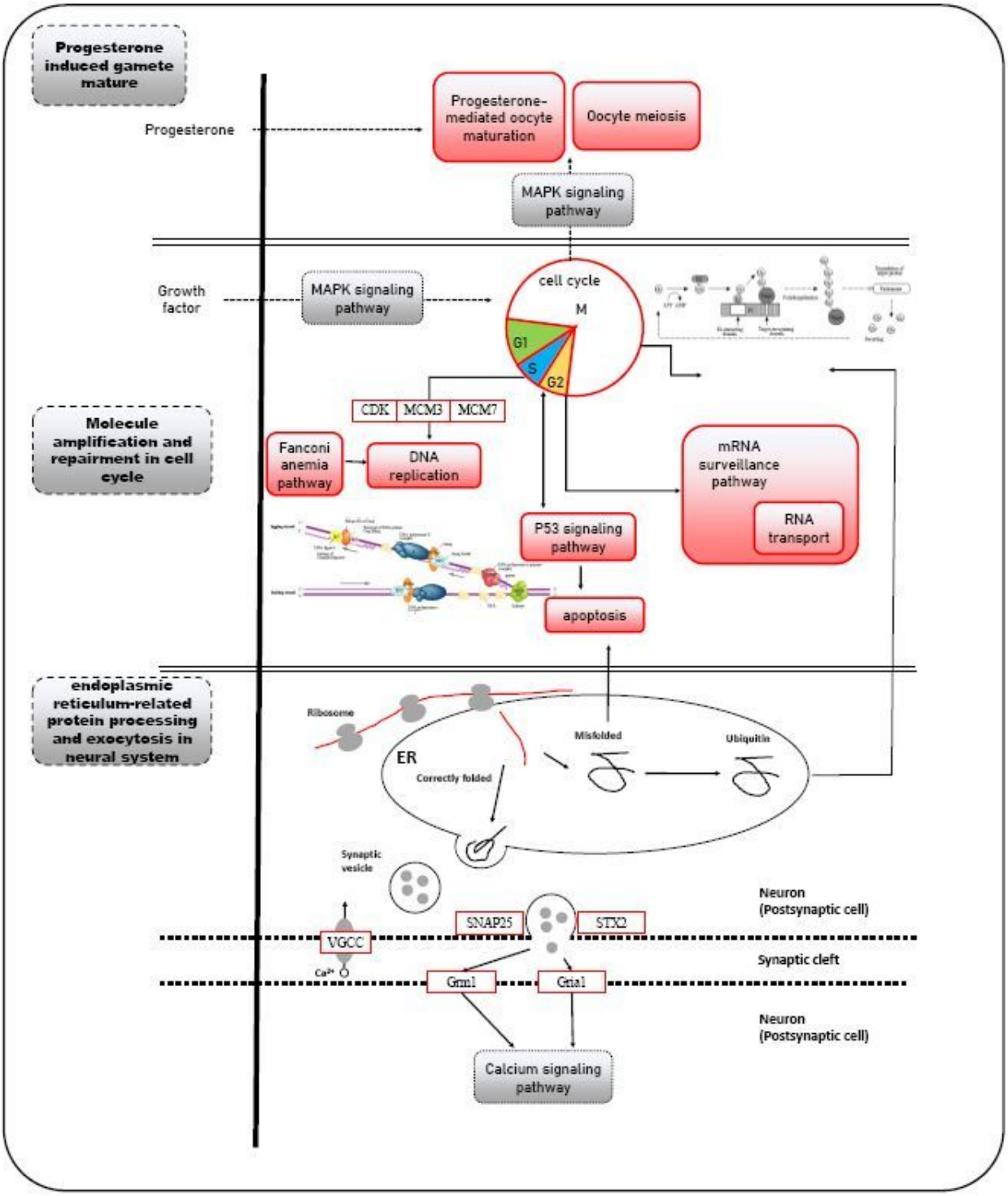

Figure 6

Regulation of pathways in M_IV vs M_V group of black rockfish. Red and green orthogon represent up and down regulated KEGG pathways and DEGs, respectively(45, 49, 82).

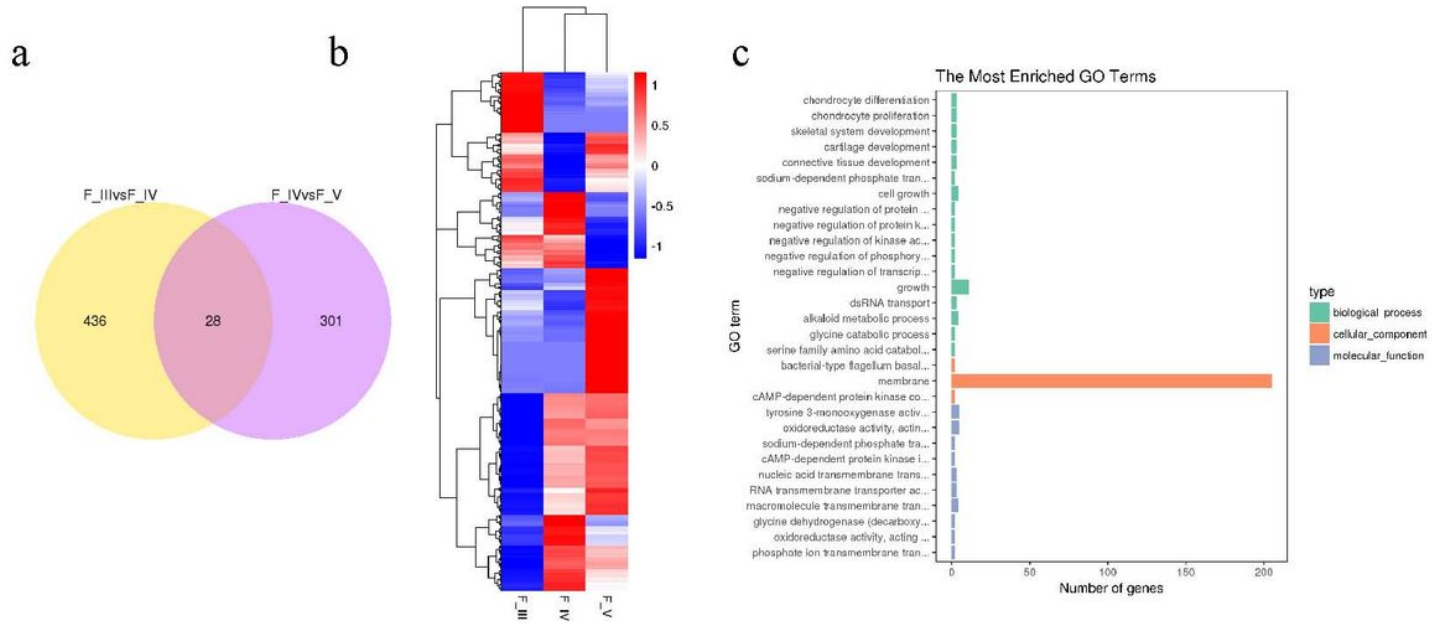

Figure 7

Identification and annotation of DEGs in different development stage in ovary of black rockfish. (a) Venn diagram of the DEGs of F_III vs F_IV and F_IV vs F_V. (b) Expression values of 765 DEGs in 3 libraries (F_III, F_IV, F_V) are presented in heat map. Red and blue colors indicate up- and down- regulated transcripts, respectively. (c) Gene ontology (GO) analysis for the 765 DEGs. The x-axis shows the number of genes in each term. The y-axis shows the specific terms. The asterisk represents the corrected p-value <0.05 in each GO term.

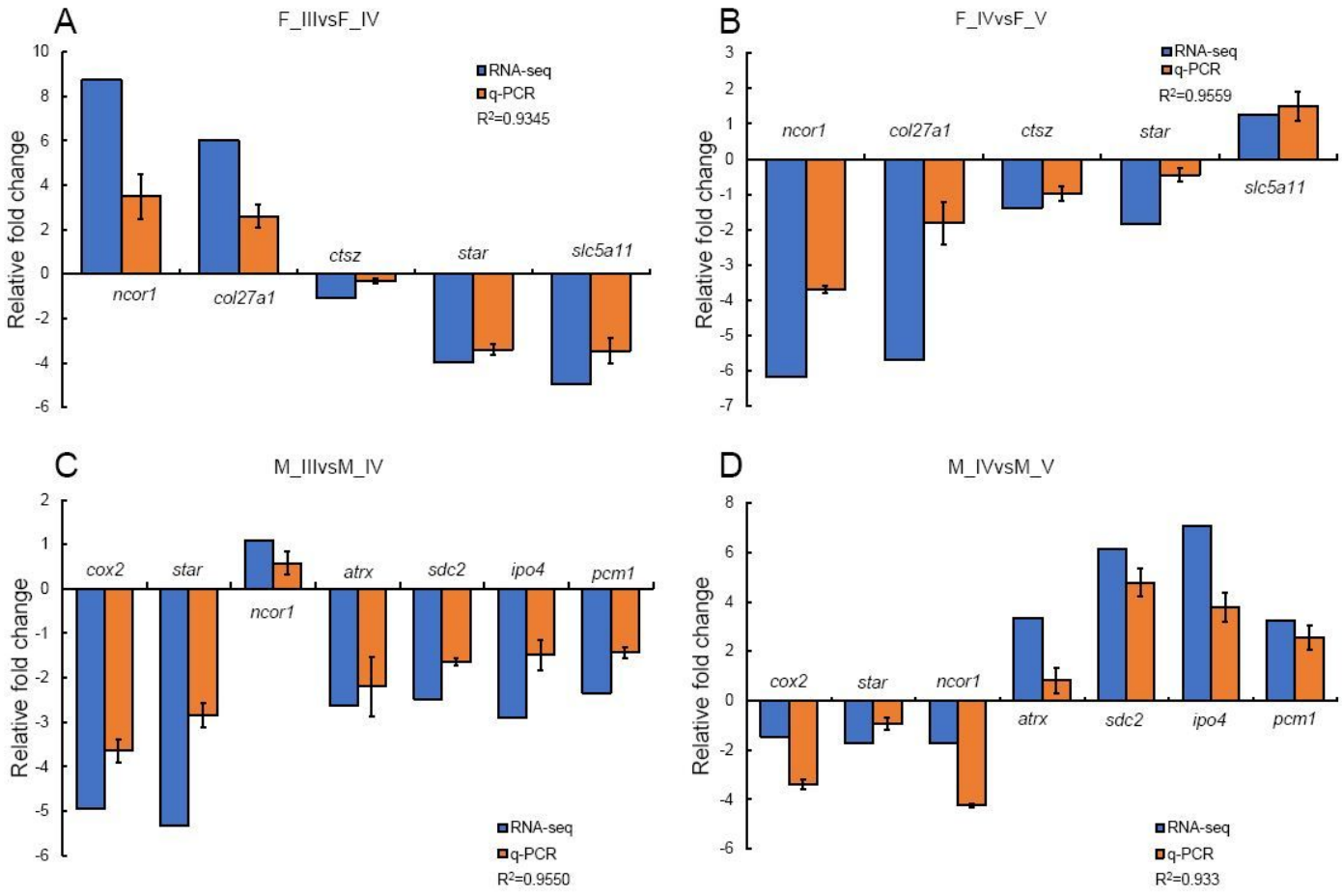

Figure 8

qRT-PCR validation of 10 differentially expressed genes generated from RNA-Seq results from gonad. The expression levels of the selected genes were normalized to the 18s gene. A: F_III vs F_IV; B: F_IV vs F_V; C: M_III vs M_IV; D: M_IV vs M_V. Gene abbreviations are: nuclear receptor corepressor 1 (ncor1); collagen alpha-1(XXVII) chain B (col27a1); cathepsin Z (ctsz); steroidogenic acute regulatory protein (star); sodium/myo-inositol cotransporter (slc5a11); cyclooxygenase 2 (cox2); transcriptional regulator ATRX (atrx); syndecan-2 (sdc2); importin-4 (ipo4); pericentriolar material 1 protein (pcm1).

Supplementary Files

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