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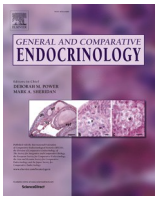

Regulation of transforming growth factor beta 1 in the ovary of ovoviviparous black rockfish (*Sebastes schlegelii*)

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ABSTRACT

Transforming growth factor beta 1 (TGF-β1), a multifunctional secreted polypeptide cytokine, has been reported to play crucial roles in pregnancy process of mammals, including immune tolerance and embryonic development. However, less is known in Pisces. Aimed to figure out the molecular mechanism underlying TGF-β1 functions, black rockfish (*Sebastes schlegelii*), an ovoviviparity teleost, which process the sperm storage and gestation periods, was employed as the research model. In the present study, we found that *tgfb1* among four *tgfb* isoforms was highly expressed and localized in ovary. The recombinant TGF-β1 (rTGF-β1) was mainly involved in immune response, signal transduction, angiogenesis and cell death by analyzing transcriptome of ovarian cells. The current results reveal the pivotal role of TGF-β1 in black rockfish ovary and provide novel insights into cytokines in the pregnancy of ovoviviparity teleost.

1. Introduction

Reproduction is one of the critical biological events in the life cycle of living organisms including fish. To achieve the success of reproduction, ensuring the existence of the species, fish have evolved reproductive strategies from oviparity, ovoviviparity to viviparity (Muchlisin, 2014). As an intermediate strategy between viviparity and oviparity, ovoviviparity shares the yolk-based nutrition of oviparity while featuring internal fertilization to viviparity (Lodé Thierry, 2012). In addition, ovoviviparous teleost also shows similar pregnancy characteristic to mammals, including the immune system variation to the semiallograft, angiogenesis, umbilogenesis and placentation. For a pregnancy to be successful, the maternal immune system needs to be precisely directed into a state of tolerance to avoid rejecting the semiallogeneic fetus (Svensson-Arvelund et al., 2015). Therefore, studies have focused on how cytokines influence the mother-fetus, and impact the success or failure of pregnancy by immune cells and cytokines coordinating with each other (Raj Raghupathy and Jroslaw Kalinka, 2008). Among the numerous cytokines, transforming growth factor beta (TGF-β) plays a considerable role in regulating the immune physiological processes of pregnancy, including essential events such as in mammals of embryonic implantation, trophoblast invasion, placental development, and fetal growth (Wen et al., 2023).

TGF-βs, conserved in the animal kingdom, is considered to have appeared from the early days of metazoan evolution, which are known in mammals (TGF-β1/2/3), in birds (TGF-β2/3/4), in amphibians (TGF-β2/5) and a novel TGF-β6 first found in gilthead sea bream (*Sparus aurata*)(Huminiecki et al., 2009; Hinck et al., 2016; Funkenstein et al., 2010). TGF-β1, the most prevalently and diversely expressed cytokine, is vital for maintaining immunological homeostasis, sustaining immune cell functions, and modulating immune cell differentiation (Clark and Coker, 1998). It has been shown that maternal first trimester TGF-β1 levels to be higher in pregnancies, which impedes maternal immune cell activity and reduces the risk of immune-mediated fetal reaction (Hernandez-Valencia et al., 2001; Shooner et al., 2005). In mice, TGF-β1 was found throughout pregnancy and affected the outcomes of pregnancy as well as increasing the fecundity rate and live birth numbers (Kay et al., 2021). Across several biological processes, TGF-β1 also serves as a multifunctional cytokine in cell proliferation, differentiation, migration, and apoptosis. TGF-β1 has been proved in both porcine and hen to mediate the states of granulose cells (Li et al., 2021; Johnson et al., 2004). However, the effects of TGF-β1 on proliferation or apoptosis are highly dependent on the cell type and context. TGF-β1 was found to inhibit the proliferation of primordial cells in fetal mouse ovary (Wang et al., 2014). In bovine, TGF-β1 promoted apoptosis of granulosa cells during time in culture and reduced entry into the proliferative

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phase of the cell cycle (Zheng et al., 2009). Besides, recombinant goldfish TGF-β induced the proliferation of a goldfish fibroblast cell line, which was the first functional report of TGF-β in teleost (Haddad et al., 2008a). TGF-β1 exerted an inhibitory on the proliferation of peripheral blood lymphocytes in grass carp (Yang et al., 2012).

Black rockfish (*Sebastes schlegelii*), which belongs to the *Sebastes* genus in the Sebastes family, has an ovoviviparous reproductive pattern. The long-term sperm storage and asynchronized gonadal development may have developed from adaptive evolution (Wang et al., 2021). In black rockfish, spermatogenesis begins in July and the sperm matures in December or the following January with individual difference. After mating, the sperms are passed into female ovary by urogenital papillae and stored in the ovary cavity during the vitellogenesis. Oocytes mature and activate the sperms for fertilization around in April. After in situ fertilization, embryonic development relies on the yolk and maternal nutrients. Following a gestation for about one month, the females give birth to fries. However, during the one-month pregnancy, the variation of immune system, angiogenesis and functional change from the ovary to the uterus-like status may affect the finial destiny of the embryos. We hypothesized that TGF-β1, as a multifunctional cytokine, plays a regulatory role in the ovary of black rockfish. To test this hypothesis, we first investigated the expression pattern and ovary localization of TGF-β1. We then performed transcriptomic analysis on ovarian cells treated with recombinant TGF-β1. Our findings demonstrated, for the first time, the function of TGF-β1 in an ovoviviparous teleost and provided evidence supporting the essential roles of cytokines in the pregnancy.

2. Materials and methods

2.1. Animals and Ethics statement

All animal experiments were reviewed and approved by the Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201) . This experiment did not involve endangered or protected species. All experiments were performed in accordance with the relevant guidelines and regulations.

Black rockfish were obtained from an aquaculture population in marine cages offshore of Penglai (37.6°N, 120.8°E), located in the northern Yellow Sea, Shandong Province, China. Prior to sampling, the fish were anesthetized with 100 ng/mL MS-222 (3-aminobenzoic methanesulfonate acid). In total, three adult females were sampled for tissue distribution analysis in December 2021. Separately, to investigate *tgfb* expression patterns across the reproductive cycle of black rockfish (Wang et al., 2021), ovarian tissues were collected from different individuals at representative stages, including previtellogenesis stage (PV, September 2021), vitellogenesis stage (V, December 2021), mature stage (M, April 2022). Additional stages from May to June were categorized based on fertilization and parturition processes, including fully mature but unfertilized stage (MM, LM), fertilized stage (F), sarcomere stage (S), before parturition stage (Pb), during parturition stage (Pd), after parturition stage (Pa)(Zheng et al., 2023; Yan et al., 2023). Three female black fishes were selected for each stage. Parts of the ovary tissue were fixed in 4 % paraformaldehyde for *in situ* hybridization.

2.2. RNA extraction, reverse transcription, and qPCR

Total RNA was extracted from various tissues and ovaries in different developmental stages in black rockfish using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Concentrations and quality of the RNA were assessed by a Biophotometer (OSTC, China) and 1 % agarose gel electrophoresis. Complementary DNA (cDNA) was prepared using the *Evo M−MLV* RT Kit with gDNA Clean for qPCR (Accurate Biology, China).

qPCR was performed using the ChamQTM SYBR Color qPCR Macter Mix (High Rox Premixed) kit (Vazyme, China) following the reagent instructions. The threshold circulation (C_T) values were measured three times for each sample, and *18 s* (KF430619.1) was selected as an internal reference gene. The relative expression level of the genes was calculated using the $2_T^{-\Delta\Delta C}$ method. The primers for qPCR are shown in Table 1.

2.3. Molecular cloning and sequence analysis

Based on the genome (CNA0000824) and transcriptome date (PRJNA573572), the open reading frames (ORFs) of *tgfb1* was predicted in black rockfish and the cloned primers for the full-length cDNA sequence are shown in Table 1. All primers used in the present study were designed by Primer 5 software (Premier, Canada). The 2 × Phanta Max Master Mix (Dye Plus) was used for cloning and ovary cDNA was used as the template. The PCR product was purified and cloned into the pCE2 TA/blunt-Zero vector (Vazyme, China) for sequencing.

The signal peptide was predicted using the signal 6.0 program (https://services.healthtech.dtu.dk/services/SignalP-6.0).The pro-peptide and

Table 1
Primers sequences used for orf cloning, ish, vector construction and qpcr.

Primers	Sequences (5' - 3')
Primers for ORF clone	
tgfb1-orf-F	ATGAAGCTGGTGGTCTTGATGC
tgfb1-orf-R	TTAGCTACACTTGCAGGACTTC
Primers for ISH	
tgfb1-ISH-F	CGCATTTAGGTGACACTATAGAA
	GCGCTCAGAGATACGGGACAGCG
tgfb1-ISH-F	CCGTAATACGACTCACTATAGGGA
	GACATGGTTGCTTTGTCATCGCCT
Primers for pET-N-His vector	
pET-*tgfb1*-F	GTAGCGGTTCCGGTTCTTCGACGG AGACGAAAGACACC
pET-*tgfb1*-R	GCTCGAATTCGATATCGGATTAGCT
	ACATTTGCACGACTTCAC
Primers for qPCR	
18s-F	CCTGAGAAACGGCTACCACAT
18s-R	CCAATTACAGGGCCTCGAAAG
tgfb1-F	TAGGGGAGACACGGGACTTT
tgfb1-R	TGCAGGTGTCTTTCGTCTCC
tgfb2-F	TGCTCTACAACAGCACGAGG
tgfb2-R	CGACTCTGTAATGGGGGTCG
tgfb3a-F	CCTGGACACTCAGTCCACAC
tgfb3a-R	GGAGCAGTAGTTGGCATCGT
tgfb3b-F	ACTGTCCCTGCCACACTTTC
tgfb3b-R	GCATCGTCAGTAGTCCGCTT
il6-F	GCTCTGTTGCTGTGTGCTC
il6-R	CCACACCTCCTCCTCACCT
tnfa-F	AAGGAGAACACAACCCTGCC
tnfa-R	AGTTGACCTGGAAAGACGCT
il17c-F	GCAGAGCAGGGTGTTTCTCA
il17c-R	CTAGGGTGAGGACTTGGCTC
dll3-F	GACCTGGAGACCGTGAACAA
dll3-R	GAGGGACGATGATGGACGAG
cd40-F	CCAGAATGTCGTCCTCCAGC
cd40-R	GTGCTTCGTTTCGTCTTGCT
il1b-F	TGATGGGCGACTTCAATCTGT
il1b-R	AGCAGCAGATAAGCGCAAGA
egr2-F	ACATCGTCTCGGGCATCTTC
egr2-R	CGCAGCCAGATGAGGAGTAA
ptgs2-F	GGAGGAGTTCTATGGGCACG
ptgs2-R	TGGGGTTTCCCATTAAGCCC
mmp13-F	ACCCAAACCCGAGGAAAGTG
mmp13-R	CCATTCAAAGCCCACATCCG
il34-F	GAAGACGCTCAACAACAGCC
il34-R	TCCAACCCCTCCACCTGTAA
il10-F	TGTCGGTTCGTGGAGTCTTT
il10-R	TTCATGGTGTGGCAGGCAA
fas-F	ACCTGTTGTCTGTGTGGAGC
fas-R	ACAGGGTTCGTCCACCTCTA
mafb-F	AAGTTCGGCGTGAAGAAGGA
mafb-R	GTATCCAGAACCCGTCGCTC
kif11-F	GGAGGTGATCGTGAAGAC CG
kif11-R	ATGGGGCAAACAACACTCCT
klc-F	CCAGAGCACACGAGAAGGAG
klc-R	AGGTTGTGTTCACTGTCGGG

mature peptide were predicted by the conserved domains search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The 3D structure of mature peptide dimerize was modeled using SWISS-Model. Multiple alignments of several species were generated by the Clustal X software. The phylogenetic tree was constructed with the neighbor-joining method using MEGA11. The values on the tree represent bootstrap scores of 1000 iterations, indicating the credibility of each branch.

2.4. In situ hybridization (ISH)

Following the manufacturer's instructions, the 2 × Phanta Max Master Mix (Dye Plus) kit was used for PCR. The PCR product was used as the template to synthesize antisense and sense RNA probes in vitro using the DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland). Ovaries at different developmental stages were fixed in 4 % paraformaldehyde followed by paraffin embedding. Serial sections (7 μm) were prepared using a microtome (Leica, Wetzler, Germany). ISH was performed as previously reported (Lyu et al., 2022).

2.5. Recombinant expression of TGF-β1

The cDNA region encoding mature peptide of TGF-β1 was amplified using primers with overlapping sequences, and the PCR product was subcloned into a pET series expression vector. The recombinant expression vector was transformed into *E. coli* Rosetta and cultured in Luria Broth (LB) medium supplemented with 100 μg/mL ampicillin and 33 μg/mL chloramphenicol. Protein expression was induced with 0.6 mM IPTG at 37 °C for 5–6 h. Following cell harvest by centrifugation, lysis was performed by sonication. The recombinant TGF-β1, expressed as inclusion bodies, was isolated from the pellet, denatured, and purified using standard washing and solubilization procedures. Refolding was achieved through gradual dilution at 4 °C to facilitate the restoration of native conformation. The solute of the fully renatured protein of TGF-β1 was transferred to PBS by ice bath dialysis. The concentration of TGF-β1 protein was determined by Bradford Protein Assay Kit (Beyotime Biotechnology, China). The protein was mixed with the SDS-PAGE Sample Loading Buffer (Beyotime Biotechnology, China) and incubated at 95℃ for 10 min to denature. The SDS-PAGE was then carried out and dyed using Coomassie Blue Fast Staining Solution (Beyotime Biotechnology, China). The remaining protein of TGF-β1 was frozen by liquid nitrogen and stored in −80℃.

2.6. Culture of ovarian matrix cells

Ovaries were collected from euthanized developing and pregnant female black rockfish. After dissection, the ovarian parietal membrane, along with embryos or oocytes, was carefully removed under a stereomicroscope. The remaining tissue was minced into small fragments and enzymatically digested with trypsin (biosharp, China) for 20 min. Digestion was terminated by adding an equal volume L15 (G-Clone, China) complete medium with 15 % fetal bovine serum (FBS) (G-Clone, China) and 1 % penicillin–streptomycin-gentamicin (Absin, China). The cell suspension was centrifuged at 400 g for 5 min, and the supernatant was discarded. The resulting pellet was resuspended in complete medium and seeded onto six-well plates. Ovarian cells were prepared from three fish and for each fish, three technical replicates were randomly assigned to wells. Cells were cultured at 25 ℃ in a CO_2-free incubator for 48 h, the L15 medium without FBS but containing 1 % penicillin–streptomycin-gentamicin concentration was used to culture the cells for 12 h. After low-transcription conditions, the complete medium with PBS (solvent control, n = 3), rTGF-β1 (final concentration: 50/125/250 ng/mL, n = 3) was used to treat cells for 6 h. Subsequently, the cells were harvested for RNA extraction.

2.7. RNA-seq analysis

RNA isolation from cell samples treated with PBS and 250 ng/mL TGF-β1 was performed as described previously. Qualities and concentrations of total RNA were evaluated by a Nanodrop (Thermo Fisher Scientific, USA) and Agilent 2100 bioanalyzer system (Agilent Technologies, USA). The RIN (RNA Integrity Number) value of each sample was above 9.0. A total of 6 sequencing libraries were constructed by the NEBNext ultra^{TM} RNA Library Prep kit for Illumina (NEB, USA) according to the manufacturer's instructions. The samples were sequenced on an Illumina Hiseq X Ten platform, and 150-bp paired-end reads were generated.

The transcriptomic data were removed reads with low quality or reads containing poly-N. The cleaned reads were aligned to the reference *Sebastes Schlegelii* genome (PRJNA516036) with histat2(Kim et al., 2015). The analysis of quantification was accomplished with the StringTie package (Pertea et al., 2016). The multiple mapped reads were removed, and the count of unique mapped reads and FPKM (Fragments Per Kilobase Per Million) were retrieved, which were standardized using previous reference (Anders et al., 2015). Statistical analysis of transcripts was based on the DESeq2 package and the differentially expressed genes (DEGs) were identified as p-value < 0.05 and $|\log_2(\text{fold change})| > 1$. DEGs annotation was mapped to the reference genome of *Sebastes schlegelii*. Thereafter, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs were performed by the clusterProfiler R package with the *p*-value threshold < 0.05(Götz et al., 2008; Kanehisa et al., 2017). The predicted protein–-protein interaction (PPI) network was analyzed and visualized by Cytoscape v3.10.1.

2.8. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way ANOVA followed by Tukey-HSD multiple range tests and differences were considered significant at $P < 0.05$. Pearson correlation analysis was performed, and the coefficient of determination (R^2) was calculated to evaluate the consistency between RNA-seq and qPCR results. All statistical processes and graphs were generated with SPSS20.0 (SPSS, USA) and GraphPad Prism 9 (GraphPad Software, USA).

3. Results

3.1. Expression patterns of tgfb isoforms in different stages

Four genes *tgfb* isoforms were identified from the genome and transcriptome of black rockfish. The qPCR results showed that the expression level of *tgfb1* was significantly increased in developing ovary and the highest level was detected when oocyte maturation (Fig. 1**A**). Based on transcriptomic data in early (PRJNA820964) and late (PRJNA886856) pregnancy, the $\log_2(\text{TPM} + 1)$ of *tgfb1* was higher than *tgfb2*, *tgfb3a*, *tgfb3b* (Fig. 1**B**) . The relative expression level of *tgfb1* was significantly lower before parturition compared to both the oocyte maturation and early pregnancy stages (Fig. 1**C**). In contrast, *tgfb2* expression remained stable across gestational and parturition stages (Fig. 1**D**). Notably, *tgfb3a* exhibited elevated expression levels throughout pregnancy and parturition (Fig. 1**E**), whereas *tgfb3b* was significantly downregulated specifically during parturition (Fig. 1**F**).

3.2. Sequence analysis of tgfb1

The cDNA sequence of *tgfb1* (Accession number: PV242391) was cloned based on black rockfish genomic data. The ORF of *tgfb1* 1161 bp in length encoded 386 amino acids. The coding region comprised a putative signal peptide spanning 57 bp, a pro-peptide region of 732 bp, and a mature peptide region of 339 bp. (Fig. 2**A**). Amino acids sequence

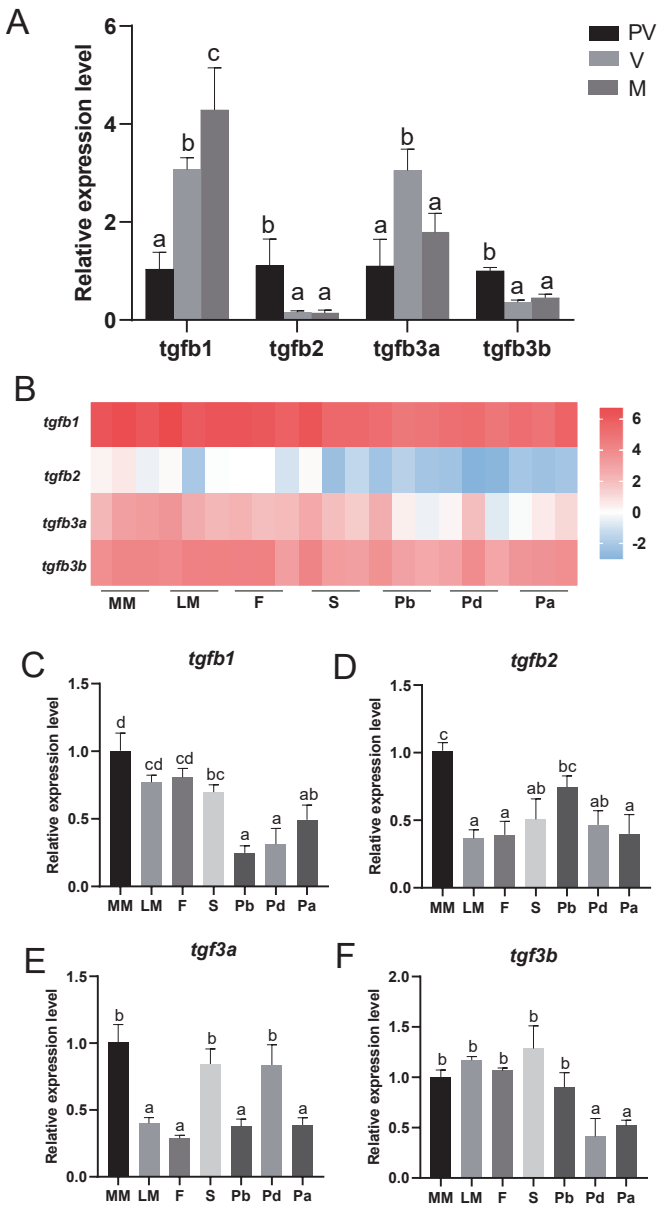


Fig. 1. (A) Relative expression levels of *tgfb* isoforms in developing ovary. The heatmap(B) and relative expression levels(C-F) of *tgfb* isoforms in different pregnancy periods. The X axis indicates different development stages in black rockfish. The Y axis indicates the relative expression normalized to *18 s* rRNA. The data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$). PV: previtellogenesis; V: vitellogenesis; M: mature stage; MM and LM: mature but unfertilized stage; F: fertilized stage; S: sarcomere stage; Pb: before parturition stage; Pd: during parturition stage; Pa: after parturition stage.

alignment indicated that the black rockfish TGF-β1 protein comprised seven α helixes and seventeen β folds (Fig. 2**B**). Phylogenetic analysis based on the amino acid sequence revealed two major branches in the neighbor-joining tree, with all teleost TGF-β1 proteins clustered together, while those from tetrapods formed a separate clade (Fig. 2**C**). In addition, nine conserved cysteine residues were identified in the mature peptide of black rockfish TGF-β1, and two monomers were predicted to form a functional homodimer (Fig. 2**D**).

3.3. Expression pattern and localization of tgfb1

Tissue distribution analysis showed that *tgfb1* was widely expressed in various tissues. In particular, *tgfb1* was highly expressed in the ovary, spleen, heart, kidney and gill, while lower expression levels were detected in liver, stomach, intestines and brain (Fig. 3**A**). As shown in Fig. 3**B**, during pregnancy in the black rockfish, the positive signals of *tgfb1* were observed in the follicular layer and embryo.

3.4. Recombinant expression of TGF-β1 and functional verification

To further study the molecular function of TGF-β1, rTGF-β1 was generated through a prokaryotic expression system (Fig. 4**A**). A single band of approximately 14.7 kDa was observed on the SDS-PAGE gel after inclusion solubilization and renaturation (Fig. 4**B**). The concentration of rTGF-β1 was 0.15 ng/mL. Primary cells from black rockfish head kidney were cultured to verify the bioactivity of rTGF-β1. rTGF-β1 significantly increased *il6* and *tnfa* expression levels compared with control group ($P < 0.05$) (Fig. 4**C, 4D**), which indicated that rTGF-β1 was functional active *in vitro*.

To understand the role of rTGF-β1 in ovary cells during developing and pregnant period, we treated ovary cells with different concentration of rTGF-β1. The *il6* and *il17c* levels were significantly increased with treated 250 ng/mL rTGF-β1(Fig. 5**)**.

3.5. RNA-seq analysis of ovarian matrix cells

The Illumina platform was applied for detecting RNA-seq in ovarian samples from four different treatments (Accession number: PRJNA1230364). After preprocessing and removing low-quality sequences, the average clean reads of the control and treatment groups in developing ovary used in subsequent analysis were 44,292,410 and 46079510, with Q30 average percentages of 94.41 % and 93.79 %. And the average clean reads of control and experimental samples in pregnant ovary were 43,527,740 and 42987626. Accordingly, the Q30 average percentage of the groups was 93 % and 93.84 %. In addition, more than 90 % average total map was totally obtained by mapping these clean reads with black rockfish genomes.

The PCA results showed twelve samples from D_control, D_TGF-β1, P_control and P_TGF-β1 groups could be well divided (Fig. 6**A**). A heatmap of differential genes expression pattern is shown in Fig. 6**B**. Differential expression analysis showed 1427 DEGs (559 upregulated DEGs and 868 downregulated DEGs) and 1362 DEGs (717 upregulated and 645 downregulated DEGs) in D_control vs D_TGF-β1 and P_control vs P_TGF-β1, respectively (Fig. 6**C, 6D**).

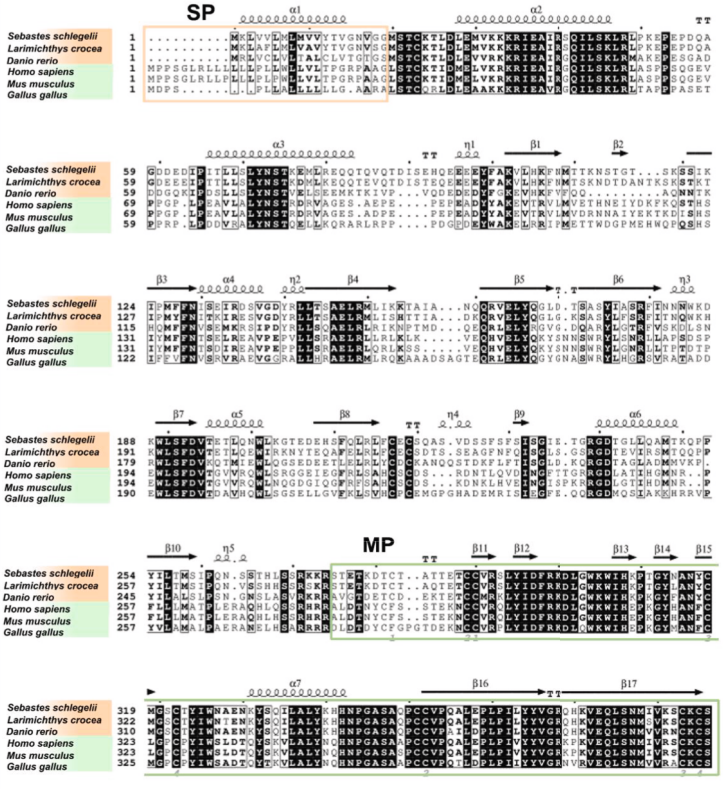
The GO enrichment analysis of DEGs were classified into biological process (BP), cellular component (CC), and molecular function (MF), respectively. Compared with control group, significantly upregulated enriched GO terms ($P < 0.01$) in developing ovary were mainly associated with immune response and signal transduction (Fig. 7**A**). And the GO analysis performed the 717 upregulated DEGs identified in P_control vs P_TGF-β1. The top 20 GO terms include immune response, cell death and signal transduction (Fig. 7**B**). The Venn graph showed that TGF-β1 treatments groups common upregulated 129 DEGs. KEGG enrichment analysis was mainly divided into three categories, including immune response (Toll-like receptor signaling pathway; C-type lectin receptor signaling pathway; RIG-I-like receptor signaling pathway; NOD-like receptor signaling pathway; Cytokine-cytokine receptor interaction), cell death (Apoptosis; Necroptosis), and angiogenesis (TGF-β signaling pathway; VEGF signaling pathway) (Fig. 8).

The PPI analysis revealed a complex network of interactions among DEGs. As shown in Fig. 9, five hub genes including *tnf*, *il10*, *cd40*, *tbx21* and *il21r* were identified for pivotal candidate genes among 129 upregulated DEGs, and *csf1r*, *lrp1* and *plek* were identified as hub genes for 109 common downregulated DEGs, respectively. In addition, eight genes were randomly selected from D_control vs D_TGF-β1 and P_control vs P_TGF-β1 for qPCR analysis to verify the gene expression patterns in the transcriptome. The results indicated that qPCR expression pattern of the selected genes was significantly correlated with the RNA-seq results

A

1	ATG	AAG	CTG	GTG	GTC	TTG	ATG	CTC	ATG	GTT	GTG	TAC	ACG	GTG	GGC	AAC	GTA	GGC	GGT	ATG
1	M	K	L	V	V	L	M	L	M	V	V	Y	T	V	G	N	V	G	G	M
61	TCT	ACG	TGT	AAG	ACG	CTG	GAC	CTG	GAG	ATG	GTA	AAG	AAA	AAG	CGC	ATT	GAG	GCC	ATC	AGG
21	S	T	C	K	T	L	D	L	E	M	V	K	K	K	R	I	E	A	I	R
121	AGC	CAG	ATC	CTC	AGC	AAA	CTG	CGC	TTG	CCA	AAA	GAG	CCT	GAG	CCA	GAT	CAG	GCT	GGA	GAC
41	S	Q	I	L	S	K	L	R	L	P	K	E	P	E	P	D	Q	A	G	D
181	GAC	GAG	GAC	ATC	CCC	ATC	ACC	CTG	TTG	TCC	CTC	TAC	AAC	AGC	ACC	AAG	GAG	ATG	CTG	AGG
61	D	E	D	I	P	I	T	L	L	S	L	Y	N	S	T	K	E	M	L	R
241	GAG	CAG	CAG	ACC	CAG	GTC	CAG	ACG	GAC	ATC	TCC	GAA	CAT	CAG	GAG	GAG	GAG	GAG	TAC	TTC
81	E	Q	Q	T	Q	V	Q	T	D	I	S	E	H	Q	E	E	E	E	Y	F
301	GCT	AAG	GTG	CTG	CAC	AAG	TTC	AAC	ATG	ACC	ACA	AAA	AAT	AGC	ACA	GGG	ACC	TCC	AAG	AGC
101	A	K	V	L	H	K	F	N	M	T	T	K	N	S	T	G	T	S	K	S
361	TCC	ATA	AAG	ATC	CCG	ATG	TTC	TTC	AAC	ATC	TCA	GAG	ATA	CGG	GAC	AGC	GTG	GGA	GAT	TAT
121	S	I	K	I	P	M	F	F	N	I	S	E	I	R	D	S	V	G	D	Y
421	CGC	CTG	CTG	ACC	AGC	GCC	GAG	CTG	CGG	ATG	CTC	ATC	AAG	AAA	ACC	GCG	ATC	GCA	AAT	CAG
141	R	L	L	T	S	A	E	L	R	M	L	I	K	K	T	A	I	A	N	Q
481	CAG	CGG	GTG	GAG	CTG	TAC	CAA	GGT	CTG	GAT	ACC	TCG	GCC	AGC	TAC	ATC	GCT	TCC	CGC	TTC
161	Q	R	V	E	L	Y	Q	G	L	D	T	S	A	S	Y	I	A	S	R	F
541	ATC	AAC	AAC	AAC	TGG	AAA	GAC	AAA	TGG	CTG	TCC	TTC	GAT	GTC	ACC	GAG	ACC	CTG	CAG	AAC
181	I	N	N	N	W	K	D	K	W	L	S	F	D	V	T	E	T	L	Q	N
601	TGG	CTC	AAA	GGG	ACC	GAG	GAT	GAG	CAT	AGT	TTC	CAA	CTT	CGG	CTG	TTC	TGT	GAA	TGC	AGC
201	W	L	K	G	T	E	D	E	H	S	F	Q	L	R	L	F	C	E	C	S
661	CAG	GCA	AGT	GTC	GAC	AGT	AGC	TTC	AGT	TTT	TCC	ATC	TCT	GGG	ATC	GAG	ACC	GGT	AGG	GGA
221	Q	A	S	V	D	S	S	F	S	F	S	I	S	G	I	E	T	G	R	G
721	GAC	ACG	GGA	CTT	TTA	CAG	GCG	ATG	ACA	AAG	CAA	CCA	CCC	TAC	ATC	CTG	ACC	ATG	TCC	ATC
241	D	T	G	L	L	Q	A	M	T	K	Q	P	P	Y	I	L	T	M	S	I
781	CCT	CAG	AAC	AGC	AGC	ACC	CAC	CTC	TCC	TCG	CGC	AAA	AAA	CGC	TCC	ACG	GAG	ACG	AAA	GAC
261	P	Q	N	S	S	T	H	L	S	S	R	K	K	R	S	T	E	T	K	D
841	ACC	TGC	ACA	GCC	ACG	ACG	GAG	ACG	TGC	TGC	GTG	CGG	AGC	TTG	TAC	ATC	GAC	TTC	AGG	AAA
281	T	C	T	A	T	T	E	T	C	C	V	R	S	L	Y	I	D	F	R	K
901	GAT	CTG	GGC	TGG	AAG	TGG	ATA	CAC	AAG	CCG	ACG	GGC	TAT	AAT	GCC	AAC	TAC	TGC	ATG	GGG
301	D	L	G	W	K	W	I	H	K	P	T	G	Y	N	A	N	Y	C	M	G
961	TCC	TGC	ACC	TAC	ATC	TGG	AAT	GCT	GAA	AAC	AAA	TAT	TCT	CAG	ATT	TTG	GCC	CTG	TAC	AAG
321	S	C	T	Y	I	W	N	A	E	N	K	Y	S	Q	I	L	A	L	Y	K
1021	CAT	CAC	AAC	CCG	GGA	GCC	TCT	GCC	CAG	CCC	TGC	TGC	GTT	CCC	CAG	GCT	CTG	GAG	CCA	CTG
341	H	H	N	P	G	A	S	A	Q	P	C	C	V	P	Q	A	L	E	P	L
1081	CCA	ATC	CTC	TAC	TAC	GTG	GGC	CGG	CAA	CAC	AAG	GTG	GAG	CAG	CTG	TCC	AAT	ATG	ATT	GTG
361	P	I	L	Y	Y	V	G	R	Q	H	K	V	E	Q	L	S	N	M	I	V
1141	AAG	TCC	TGC	AAG	TGT	AGC	TAA													
381	K	S	C	K	C	S	*													

B



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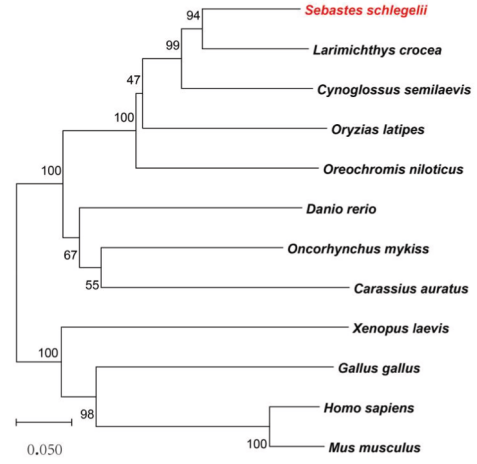

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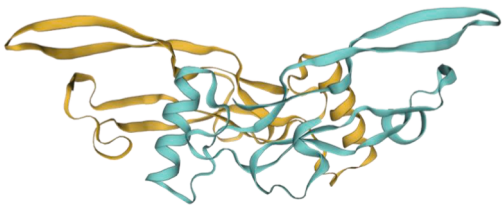

(caption on next page)

Fig. 2. (A)Nucleotide and amino acid sequence of TGF-β1 in black rockfish. "—" indicates signal peptide. RKKR is the furin cleavage site. Gray and yellow markers are LAP pre-peptide and mature peptide. Blue indicates cysteine. (B)The sequence alignment of TGF-β1 in black rockfish (*Sebastes schlegelii*), large yellow croaker (*Larimichthys crocea*), zebrafish (*Danio rerio*), human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*gallus gallus*). (C)Phylogenetic tree of TGF-β1 in black rockfish and other species. Data was resampled with 1,000 bootstrap replicates. The Genebank accession numbers are as follows: *Larimichthys crocea* (KAE8294992.1), *Cynoglossus semilaevis* (XP_008307032.1), *Oryzias latipes* (XP_004075270.1), *Oreochromis niloticus* (XP_025753606), *Danio rerio* (AAO60240), *Oncorhynchus mykiss* (CAA07707.1), *Carassius auratus* (ABU55371), *Xenopus laevis* (NP_001081330), *Gallus gallus* (AAA49089.1), *Homo sapiens* (CAA29283.1), *Mus musculus* (AAA37674). (D)Three-dimensional structure prediction of TGF-β1 mature peptide in black rockfish. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

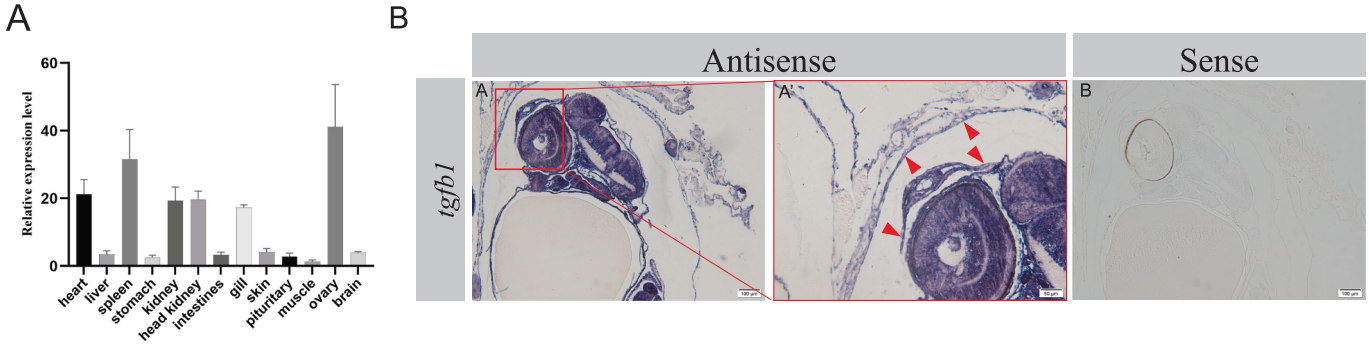

Fig. 3. (A) Relative expression levels of *tgfb1* in different organs (heart, liver, spleen, kidney, head kidney, intestine, gill, skin, pituitary, white, muscle, ovary, brain). (B) The localization of *tgfb1* in pregnant ovary of black rockfish via *in situ* hybridization. The red arrows indicate positive signals. Scale bars (A, B) = 100 μm. Scale bars (A') = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($R^2 = 0.9813$ and $R^2 = 0.9804$) (Fig. 10).

4. Discussion

As the major member of TGF-β family, TGF-β1, which evolutionarily emerged in deuterostomes and was widely found in vertebrates (Pang et al., 2011), has been reported in mammals, birds, amphibians, and many teleost, such as plaice (*Pleuronectes platessa*), sea bream (*Sparus aurata*) and goldfish (*Carassius auratus*)(Laing et al., 2000; Tafalla et al., 2003; Haddad et al., 2008b). In black rockfish, four TGF-β isoforms were identified in genome and transcriptome. Among which, the expression level of *tgfb1* mRNA was significantly increased in the developing ovary. In mammals, TGF-β, a local ovarian paracrine factor and a peptide growth factor has been reported to play important roles in the development of ovary (Drummond, 2005). Impaired ovarian function, such as oocyte incompetence, was found in *tgfb1* null mutant mice (Ingman and Robertson, 2009). TGF-β1 stimulated synthesis of steroids in granulosa cells by up-regulating expression of *cyp11a1* and *hsd3d* in rat ovary (Chen et al., 2008).TGF-β1 was detected in follicles at all stages of development and enhanced the expression of *fshr*, suggesting TGF-β1 may promote vitellogenesis in zebrafish (Kohli et al., 2003, 2005).In humans, TGF-β is highly expressed in the early stage of pregnancy, which is involved in embryo implantation, immune tolerance regulation and placental development, but its expression decreases in the late pregnancy (Wen et al., 2023). The expression profile of *tgfb1* was similar with human, which showed a pivotal physiological role during pregnancy in the ovary of black rockfish. Therefore, we identified TGF-β1 in black rockfish, which shared all the features characteristic of TGF-β superfamily, including signal peptide, pro-peptide and a mature peptide (Tzavlaki and Moustakas, 2020). Multiple sequence comparison and the phylogenetic tree showed that TGF-β1 congregated with teleost and differed from mammals. These results suggested that TGF-β1 of black rockfish was an evolutionarily conserved of secreted polypeptide factor.

In common carp (Zhan and Jimmy, 2000) and grass carp (Yang and Zhou, 2008), *tgfb1* mRNA was highly expressed in head kidney and spleen, indicating a close association between TGF-β1 and fish immunity. Similarly, in black rockfish, *tgfb1* was also highly expressed in the immune organ, supporting its role as an immunoregulatory cytokine. In addition, strong positive signals of *tgfb1* were detected in the ovary through ISH. The high expression of *tgfb1* in ovarian tissue may be related to the presence of sperm stored within the ovary, which was confirmed in the transcriptome. In the transcriptome of TGFβ1-treated developing ovarian cells, the physiological process of GO function enrichment was mainly immunity. In mice, TGF-β acted as a stimulating factor and transmitted signals activating the immune system to response the sperm (Govinden and Bhoola, 2003). High concentrations of TGF-β1 can regulate female immune tolerance to sperm in humans (Yang et al., 2021). After mating, the ovary will have an inflammatory response to sperm, and TGF-β1 promoted immune maternal tolerance to antigen (sperm), as has been demonstrated in cattle (Odhiambo et al., 2009). In black rockfish, sperm are stored in the ovary after mating. As foreign antigens, sperm can trigger an immune response in the female. Our findings suggest that TGF-β1 may contribute to maternal immune tolerance in black rockfish.

During normal pregnancy, as a multifunctional cytokine, TGF-β1 is widely involved in the regulation of immune cell function and plays an indispensable role in fetal-maternal immune tolerance (Kang et al., 2016). In black rockfish, the pregnant ovary exhibited multiple response to TGF-β1 stimulation. According to transcriptomic results, the biological processes including immune response and signal transduction accounted for the top 20 GO terms. The immune responses involved the activity of chemokines (*cxcl6*, *cxcl12*, *cxcl13*), cytokines (*il6*, *il10*, *il17*), and their corresponding receptors. Among them, IL10 was central to the polarization of homeostatic molting macrophages, and IL10 deficient mice had a significantly increased rate of spontaneous abortion (Chaouat et al., 2005). TGF-β1 induces chemokines production in peripheral blood monocytes and repairs inflammatory damage (Wahl et al., 1987). In humans, IL17a secretion by the placental macrophages participated in the morphogenetic events associated with placental development (Pavlov et al., 2018). M2 macrophages promoted cell homeostasis, trophoblast invasion, and migration by secreting TGF-β1 (Wen et al., 2023). Furthermore, KEGG analysis of TGFβ1-treated ovarian matrix cells focused on immune-related pathways. Notably, cytokine-cytokine receptor interaction is involved in gestation and hinders the process of pregnancy in case of dysregulation or uneven expression (Meyyazhagan et al., 2023). Toll-like receptor (TLR) signaling pathway has been implicated in the regulation of ovulation, fertilization, gestation and parturition in females (Kannaki et al., 2011).

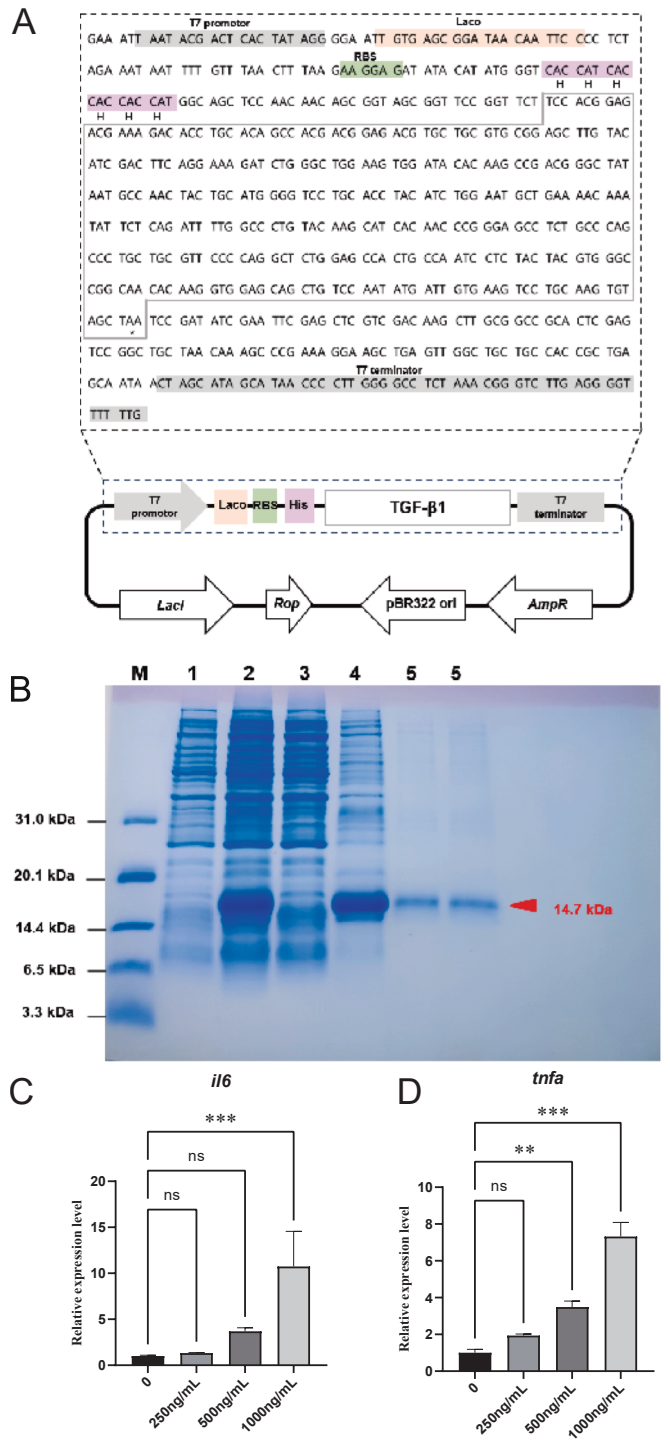

Fig. 4. (A) The pET-His-TGFβ1 vector information. (B) SDS-PAGE analysis of rTGF-β1. M: Marker. 1: Protein before IPTG induction. 2: Protein after IPTG induction. 3: Protein in the supernatant after IPTG induction. 4: Protein in the precipitate after IPTG induction. 5: rTGF-β1 after dilution and renaturation (14.7 kDa). (C-D) The relative expression level *il6* and *tnfa* in head kidney cells treated with 250, 500 and 1000 ng/mL rTGF-β1 (n = 3). The X axis indicates different treatments: control, 250 ng/mL, 500 ng/mL, and 1000 ng/mL. The Y axis indicates the relative expression normalized to *18 s* rRNA. Two and Three asterisks indicate significant difference ($P < 0.01$ and $P < 0.001$, respectively).

In mice, TLR4-null mutants exhibited impaired reproductive outcomes after allogeneic mating, with reduced pregnancy rate (Chan et al., 2021). In the present study, the regulatory pathway of TGF-β1 on developing and pregnant ovaries were similar, suggesting that TGF-β1 may serve as a key modulator of maternal immune tolerance during gestation.

TGF-β1 also play a role in angiogenesis during pregnancy by modulating several signaling pathway, including the MAPK, TGF-β, Apelin and VEGF signaling. These pathways are regulated by multiple growth factors, such as FGF、VEGF and TGF-β. Different components of the TGF-β signaling pathway work in a coordinated manner during in vivo vascular formation, and aberrant TGF-β signal transduction underlies various vascular diseases (Goumans et al., 2003). FGF-2 has been found to contribute to angioblast generation and vessel formation in quail/chick chimeras (Cox and Poole, 2000). VEGF, a prominent endothelial-specific growth factor, is known for its roles in enhancing vascular permeability and supporting angiogenesis (Dai and Rabie, 2007). In human, TGF-β1 modulates placental vascular permeability and angiogenesis by regulating VEGF production in trophoblasts during early pregnancy, thereby facilitating embryo implantation and placental formation (Chung et al., 2000). Embryos of TGF-β1 knockout mice displayed hematopoietic dysfunction and disrupted yolk sac vascular development (Dickson et al., 1995). TGF-β1 and different TGF-β1 receptor system have major roles in embryonic vascular morphogenesis and in the establishment and maintenance of vessel wall integrity (Goumans et al., 2003). During gestation in the black rockfish, the embryo surface was covered with rich network of capillaries (Zheng et al., 2023), and approximately 40 % of the energy required for development was supplied by the maternal system (Boehlert et al., 1986). Together, these findings support the potential role for TGF-β1 in contributing to ovarian angiogenesis and embryonic development during pregnancy in black rockfish. However, the mechanisms by which TGF-β1 regulates angiogenesis, as well as its specific effects during gestation, require further experimental validation.

In addition to immune response and angiogenesis induced by a series of chemokines, cytokines and their receptors, cell death was also induced by TGFβ1-treatment in ovary. Apoptosis played a crucial role during embryo implantation in rodents where morphological characteristics of apoptosis were observed in endometrial epithelial cells at the embryo implantation site (Shooner et al., 2005). In humans, TGF-β1 treatment of endometrial rat stromal cells has been shown to induce apoptosis, suggesting that TGF-β1might be involved specifically in the control of apoptosis in the uterus during pregnancy (Moulton, 1994).In black rockfish, TGF-β1 activated apoptosis-related genes and pathways in ovary. IL6, forms a positive feedback regulatory loop with TGF-β1, regulating GC apoptosis in pig ovaries (Maeda et al., 2007). The apoptotic effect of TGF-β1 in bovine MEC was mediated by IGFBPs and occurred through IGF sequestration (Gajewska and Motyl, 2004). In follicular dendritic cells, TGF-β has been found to regulate *fas* and *caspase-8* expression and thus control programmed cell death (Park et al., 2005). IL10 upregulated the expression of *fasl* in early gestation trophoblast cells and promoted immune protection (Aschkenazi et al., 2002). TGF-β1 participated in cell death either indirectly through cytokines or directly by regulating apoptotic genes, with significant enrichment of necroptosis, apoptosis and phagosome pathways, indicating the regulation of TGF-β1 maintained homeostasis in ovary. Meanwhile, regulation of cell death pathway played a pivotal role in coordinating multiple aspects of ovarian development and functional transitions. Follicles, the functional unit of ovary, atresia depend predominantly on the apoptosis of GCs (Zhou et al., 2019). Formation of preovulatory follicle involves a balance between survival and death of the oocytes and the surrounding follicular cells (Johnson, 2003). As an ovoviviparous teleost, cell death may contribute to the transition from the ovary to the uterus-like status after in situ fertilization in black rockfish.

In summary, TGF-β1 plays an indispensable role in the developing and pregnant process of ovary in black rockfish. For one thing, TGF-β1 adjusted chemokines and inflammatory cytokines to participated in the balance of immune system in the mother-fetus interaction. For another, TGF-β1 was involved in angiogenesis by regulating key angiogenic

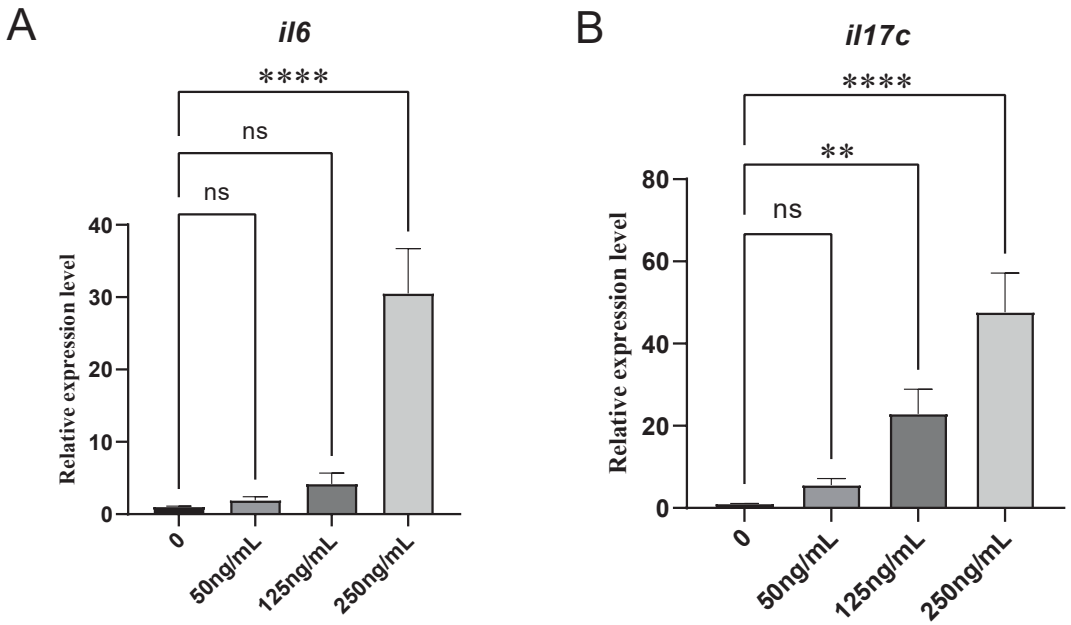

Fig. 5. The relative expression level *il6* and *il17c* in pregnant (A) and developing (B) ovary cells treated with rTGF-β1 (n = 3). The X axis indicates different treatments. The Y axis indicates the relative expression normalized to *18 s* rRNA. Two and four asterisks indicate significant difference ($P < 0.01$ and $P < 0.0001$, respectively).

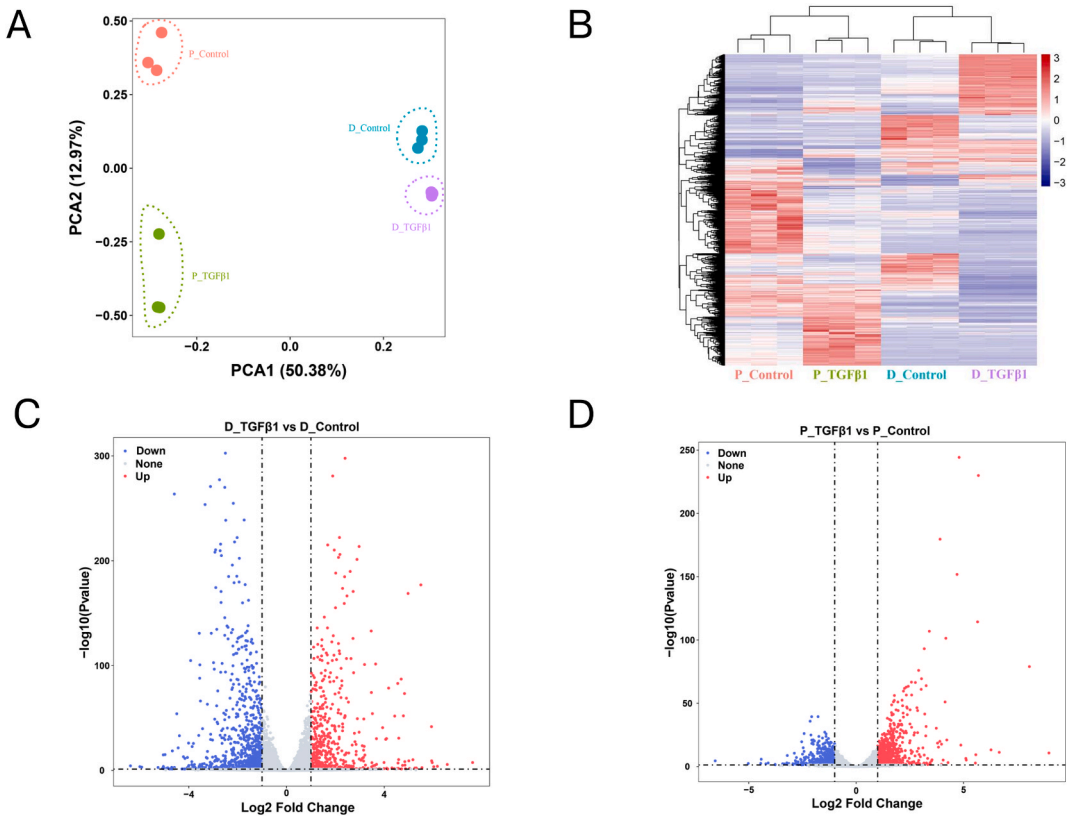

Fig. 6. (A) The PCA analysis of the control and TGF-β1 groups. (B)Heat map of differentially expressed genes (DEGs). The color in red or blue is marked up and down. (C-D) The Volcano plots for control vs TGF-β1 group. D: RNA-seq from developing ovary cells. P: RNA-seq from pregnant ovary cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

factors and pathways. Additionally, TGF-β1 also regulated cell death-related genes to transition the ovary to a uterus-like status during gestation. These results not only revealed the diverse functions of TGF-β1 but also provided additional mechanistic insights for further study of pregnancy regulation in black rockfish.

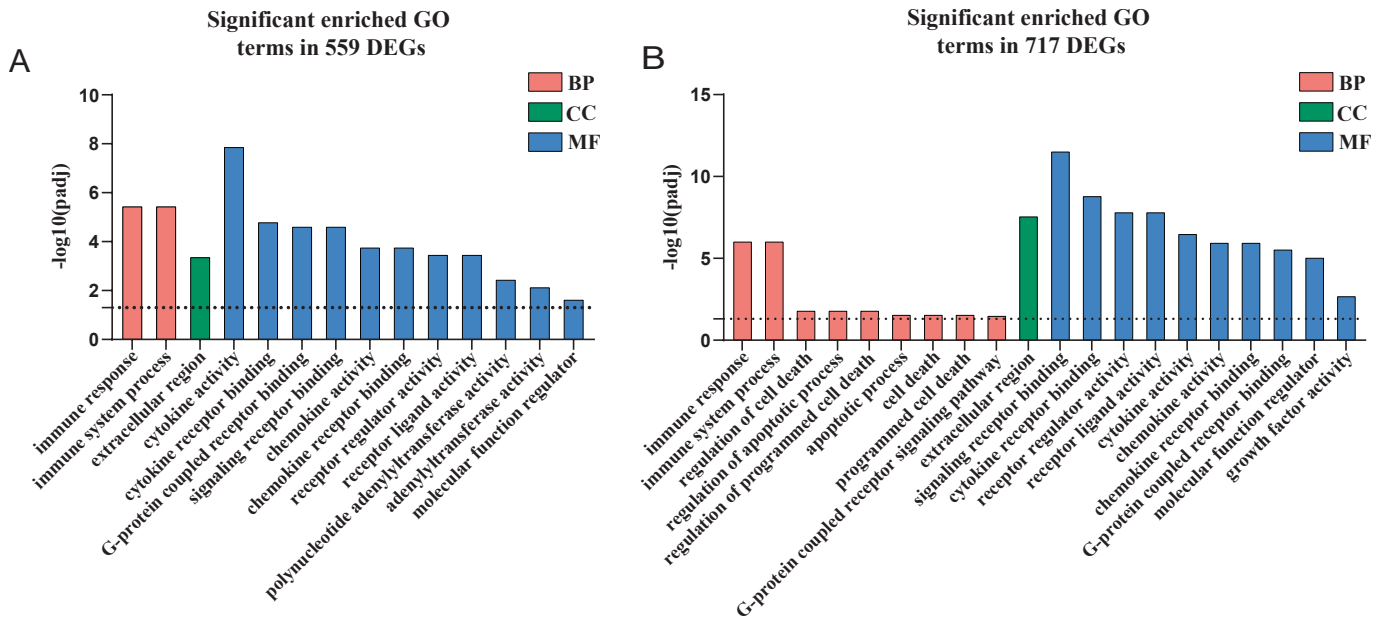

Fig. 7. (A)The top 14 GO terms of the 559 DEGs from control and TGF-β1 groups in developing ovary cells. (B) The top 20 GO terms of the 717 DEGs from control and TGF-β1 groups in pregnant ovary cells. BP: biological processes, CC: cell components, MF: molecular functions.

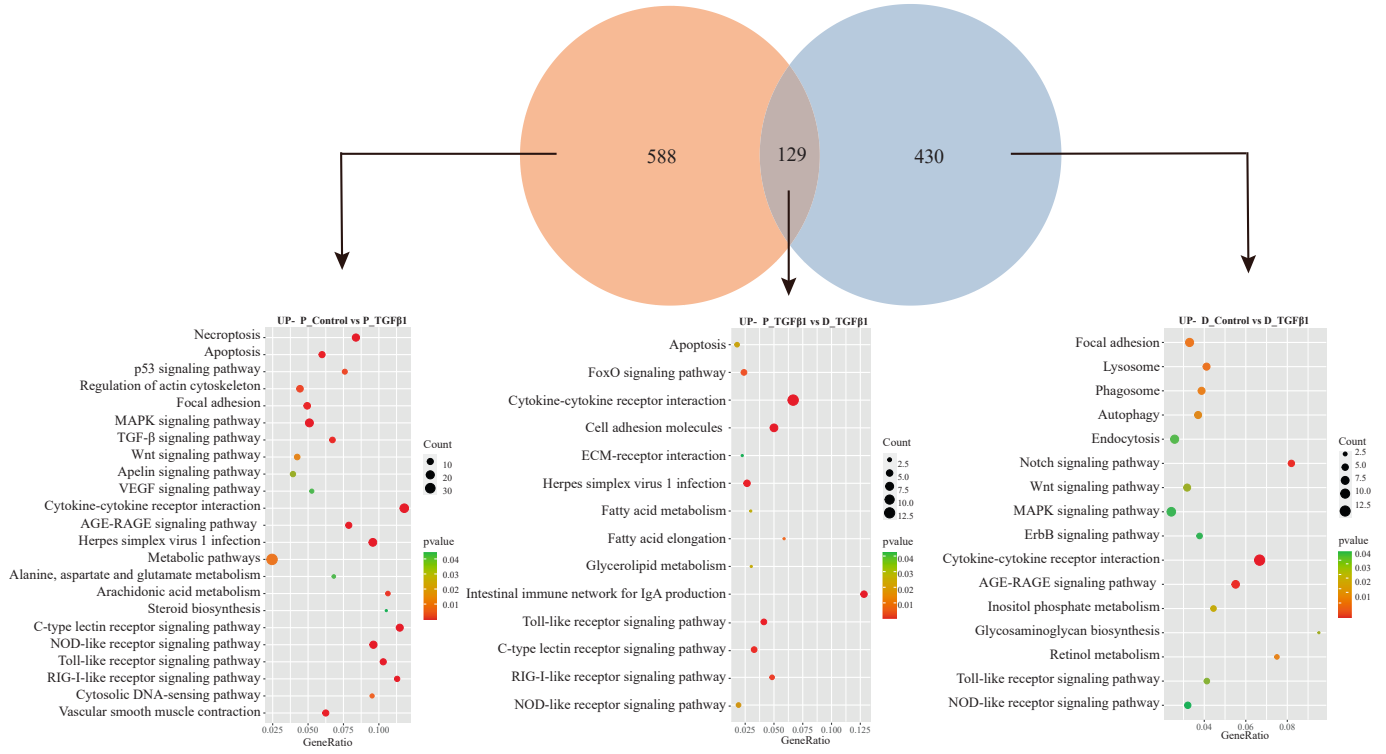

Fig. 8. Venn diagram of up-regulated DEGs in developing and pregnant ovary cells treated with rTGF-β1 and related KEGG enrichment pathway.

CRediT authorship contribution statement

Xiao Jing: Writing – original draft, Validation, Investigation, Data curation. **Likang Lyu:** Software, Formal analysis. **Chenpeng Zuo:** Software, Methodology. **Jianshuang Li:** Visualization, Conceptualization. **Xiaojie Wang:** Resources, Investigation. **Jing Yang:** Methodology, Conceptualization. **Tianyu Jiang:** Validation, Methodology. **Yun Li:** Writing – review & editing, Methodology. **Haishen Wen:** Writing – review & editing, Methodology. **Xin Qi:** Writing – review & editing, Supervision, Funding acquisition.

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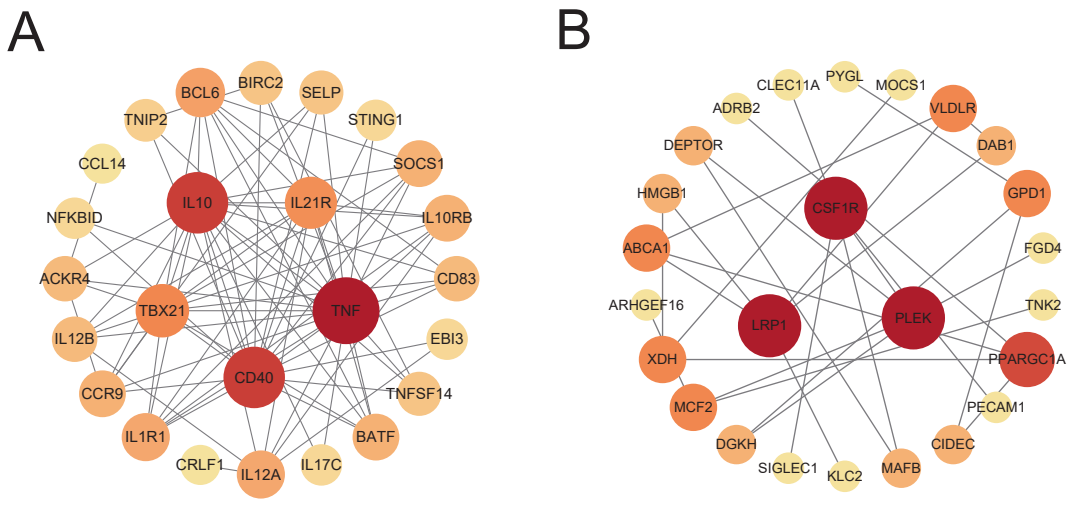

Fig. 9. Protein-protein interaction networks of up-regulated (A) and down-regulated (B) DEGs following rTGF-β1 treatment. Node colors indicate betweenness centrality, with candidate hub genes at the center of the network.

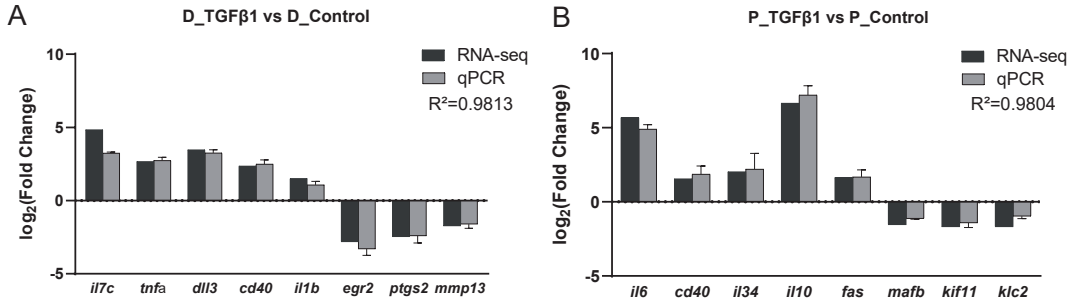

Fig. 10. Pearson correlation analysis of relative fold changes between qPCR and RNA-Seq for DEGs. (A) DEGs from developing ovary cells. (B) DEGs from pregnant ovary cells.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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