Transcriptome analysis of discus fish (Symphysodon haraldi) skin and brain to identify genes involved in 'milk' secretion during parental care

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Abstract

The discus fish Symphysodon spp., an Amazonian cichlid, employs an unusual parental care behavior where free-swimming fry feed on parental epidermal mucus after hatching. However, little is known about the mechanism by which discus secrete 'milk' and the genes involved. In order to study the unique behavior of discus fry feeding on parental skin mucus on the molecular level, transcriptome sequencing was performed on the skin and brain of female discus. Through the analysis of skin transcriptome sequencing data, 228 differentially expressed genes were obtained by comparing parental with non-parental fish, including 126 up-regulated genes and 102 down-regulated genes. For the brain, 86 differentially expressed genes were obtained including 71 up-regulated genes and 15 down-regulated genes. Through the analysis of pathway in the skin, 7 metabolic pathways were obtained: arachidonic acid metabolism pathway, adhesion pathway, apoptosis, steroid biosynthesis, tuberculosis, P53 signaling pathway, serotonergic synapse, which were related to 10 differentially expressed genes: JUNB, MRC, DPP3, CASP3, PPID, ITGA11, ALOXE3, HBE, PTPRJ, GALE. Meanwhile, the analysis of pathway in the brain, 20 metabolic pathways were obtained e.g., estrogen signaling pathways, inflammatory medlator regulation of TRP channels, non-small cell lung cancer, vascular smooth muscle contraction, which were related to 9 differentially expressed genes: PRKCD, H1-5, EDNRB, LAPTM, FOXB, OTX2, NRIF2, SOX1 and HBE.

Introduction

In mammals, offspring have access to milk, a substance rich in a range of nutritious and non-nutritious factors that are essential for the survival of the developing neonate (Jiang et al., 2020; Lu et al., 2019). Newborn ungulates such as ruminants rely on the ingestion of a sufficient amount of good-quality colostrum to acquire passive immunization and thus protecting against infectious diseases (Ahmad et al., 2007; Kessler, Pistol, Bruckmaier, & Gross, 2020). The first mammalian milk, colostrum, contains non-nutritional substances such as proteins, peptides and steroids needed by the newborn mammal and that are absorbed through the gut of the neonate. These substances play a role in development, in endocrine and in additional physiological functions, and in the immune system of the neonate (Ahmad et al., 2007; Buckley, Val, & Sloman, 2011). Feeding maternal colostrum has been shown to increase absorption of nutrients such as glucose and improve intestinal morphology and absorption of nutrients compared with formula (Ahmad et al., 2007; RH Drent, 1980). Offspring fed by parents grow and mature faster (Beekman, Thompson, & Jusup, 2019). Sometimes parental care, a prevalent behavior in teleost fishes, increases offspring survival. A behavior system for providing food for young is one of the more significant evolutionary traits shared among a wide range of taxa (Ahmad et al., 2007; Holbrook, 2011). *Boulengerula taitanus* is a direct-developing, oviparous caecilian, the skin of which is transformed in brooding females to provide a rich supply of nutrients for the developing offspring (Kupfer et al., 2006). bi-parental care in a teleost, i.e. mucus-provisioning behaviour in the scale-

eating cichlid*Perissodus microlepis* endemic to Lake Tanganyika (S. Satoh et al., 2019). American cichlids, such as substrate incubators and mouth incubators, have parental behavior (S. Satoh, Tanoue, Ruitton, Mohri, & Komatsu, 2017).

The discus fish *Symphysodon spp* ., an Amazonian cichlid, also employs an unusual parental care behavior where free-swimming fry feed on parental epidermal mucus after hatching (Ahmad et al., 2007; Buckley et al., 2010; Holbrook, 2011). Some study reports that circumstantial evidences indicate that both sexes of parents care for their offspring and provide their body mucus as food for fry when fry begin to freely swim (Ahmad et al., 2007; Shun Satoh, Tanoue, & Mohri, 2018). In discus fish, mucus provisioning is essential for offspring survival, and offspring depend on parental mucus more than any other species that provide mucus to their offspring (R. DeAngelis, Dodd, Snyder, & Rhodes, 2018). In the past many studies have focused on the behavioral characteristics of mucus feeding in the discus (Buckley et al., 2011). For example, the manner in which the number of fry influences the costs and benefits of mucus provisioning in discus fish, found that fry grew more rapidly when they were raised with parents than when raised without parents (Shun Satoh et al., 2018). Some parts also studied the composition of Midas cichlid (*Cichlasoma citrinellum*) mucus and found the amount of rich in protein, prolactin, growth hormone and so on in the mucus (Ahmad et al., 2007; Schutz & Barlow, 1997). However, there are few regulatory mechanisms involved in the secretion of milk by the skin, only test the role of hormone prolactin (PRL) in brood care behavior of the cooperatively breeding cichlid (*Neolamprologus pulcher*) (Ahmad et al., 2007; Bender, Taborsky, & Power, 2008). PRL mRNA was determined in the pituitary glands of breeders of both sexes, helpers that showed brood care behavior and nonbreeding fish as controls, but found no evidence that elevated levels of PRL are directly involved in the regulation of brood care behavior in this species (Ahmad et al., 2007; Bender et al., 2008).

The brain plays a critical role in upstream regulation of processes central to mating effort, parental effort (Bentz, Rusch, Buechlein, & Rosvall, 2019; Tavares et al., 2019). For breeding animals, rapidly shifting social and physical demands may lead to especially critical physio-logical, behavioral, and life history trade-offs (Ahmad et al., 2007; Robert, 1972; Stiver & Alonzo, 2009). Understanding how species and behaviors are coordinated among and within individuals is of keen interest in social neuroscience, especially as behavioral variation corresponds to variation within the brain (R. DeAngelis et al., 2018). Animals shift to parenting while also maintaining their own energy reserves (R Drent & Daan, 1980). The central nervous system plays a critical role in upstream regulation of these processes. The nonapeptides arginine vasopressin (AVP) and oxytocin (OT), as well as their non-mammalian homologs arginine vasotocin (AVT) and isotocin (IT), have been implicated as key neuromodulators in a variety of social behaviors, including parental care. AVP/AVT and OT/IT neuron cell bodies reside primarily within the preoptic area of the hypothalamus (POA), and project widely throughout the brain (R. DeAngelis et al., 2018). In prairie voles, AVP injections into the lateral septum, a brain region known to be involved in mediating behavioral acts related to offspring care (Dulac, O'Connell, & Wu, 2014; Wang, Ferris, & De Vries, 1994). Among teleosts, the anemonefish (*Amphiprion ocellaris*) presents an exciting opportunity for exploring neuroendocrine regulation of male parental care. *A. ocellaris* lives in relatively small and simple social groups, where pair bonds and social hierarchies are established long before mating occurs. Therefore, there is no active courtship, nest building or intraspecific aggressive interactions co-occurring during high levels of parental care, enabling the underlying regulatory mechanisms to be more specifically extricated (R. DeAngelis, Gogola, Dodd, & Rhodes, 2017; R. S. DeAngelis & Rhodes, 2016; Iwata, Nagai, & Sasaki, 2010; Ji, Long, Briody, & Chien, 2011). So, do discus fish have similar brain regulation mechanisms?

The case of parental care in fish is indeed a fascinating model system for answering a variety of questions about the cost of reproduction and the parent–offspring conflict. In order to further reveal the regulatory mechanism of discus lactation, we performed transcriptome analysis on the skin and brain tissues of parental females and non-parental females. This research through the transcriptome sequencing technology to discus fish skin stage in non-parental females (NP_S) and parental females (P_S), analysis and screening of the skin in NP_S and P_S about differentially expressed genes. The discus fish brain stage as the same as skin, discus fish brain stage in non-parental females (NP_B) and parental females (P_B). For the future of discus tending a special behavior provide theoretical basis for further research. This study will help us to understand the

mechanism underlying discus lactation and identify genes related to the lactation of discus fish and finally propose effective strategies to breed child for better control of discus lactation.

Materials and methods

Experimental fish

The breeding fish of discus is provided by the aquarium lab of Shanghai Ocean University. Six pairs of breeding discus were taken from the same litter of two breeding discus. These 6 pairs of breeding fish were raised in 6 glass tanks of the same size. The tank size was 40×40×40cm, and the effective water body was 40×40×30cm. All aquatics tanks are kept continuously aerated for 24 hours, and the temperature can be adjusted by the air in the aquatics workshop. During the experiment, the dissolved oxygen in the water in the aquaculture tank was kept above 5 mg/L and was close to saturation. The water temperature was 28.5 ± 0.3 and pH 6.1 +- 0.2. During the experimental culture, the light was from 8:00 to 20:00 every day to ensure the illumination for 12 hours every day. Feeding frozen blood worms twice a day to the breeding fish accounts for 6%˜7% of the body weight of the breeding fish. Feeding time is 8:00 and 18:00 respectively. Clean the aquiculture tank every day and replace 1/4 volume of the effective water body. Wait for the breeding fish natural reproduction.

Sample collection

The skin of three groups of discus females in the parental females (their child swim for one week, P_S (1,2,3)) and the skin of three groups of discus females in the non- parental females (the fifth day after swimming for two weeks, NP_S (1,2,3)) were put into a centrifuge tube and temporarily frozen in liquid nitrogen. After that, they were put into a refrigerator at -80 for long-term preservation and waiting for treatment.

The brain of three groups of discus females in the parental females (their child swim for one week, P_B (1,2,3)) and the brain of three groups of discus females in the non- parental females (the fifth day after swimming for two weeks, NP_B (1,2,3)) were put into a centrifuge tube and temporarily frozen in liquid nitrogen. After that, they were put into a refrigerator at -80 for long-term preservation and waiting for treatment.

RNA isolation, Library preparation, and Illumina Novaseq 6000 Sequencing

Transcriptome sequencing was performed by Meiji biotechnology co., LTD (SRA: SRP253018). Total RNA was extracted from the discus fish skin and brain tissue using TRIzol(r) Reagent according the manufacturer's instructions (Invitrogen, Carlsbard, CA, USA) and genomic DNA was removed using DNase I (TaKara). Then the integrity and purity of the total RNA quality was determined by 2100 Bioanalyser (Agilent Technologies, Inc., Santa Clara CA, USA) and quantified using the ND-2000 (NanoDrop Thermo Scientific, Wilmington, DE, USA). Only high-quality RNA sample (OD260/280= 1.8˜2.2, OD260/230[?]2.0, RIN[?]8.0, 28S:18S[?]1.0, >2μg) was used to construct sequencing library. RNA purification, reverse transcription, library construction and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer's instructions (Illumina, San Diego, CA).The discus fish skin and brain RNA-seq transcriptome libraries were prepared using Illumina TruSeqTM RNA sample preparation Kit (San Diego, CA). Total RNA was separated by oligo-dT-attached magnetic beads and then fragmented by fragmentation buffer. Taking these short fragments as templates, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). Then the synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (New England Biolabs, Boston, MA) for 15 PCR cycles. After quantified by TBS380, two RNAseq libraries were sequenced in single lane on an Illumina Novaseq 6000 sequencer (Illumina, San Diego, CA) for 2×150bp paired-end reads.

De novo Assembly and Annotation

The raw paired end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep

) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then clean data from the samples (skin and brain) were used to do de novo assembly with Trinity (http://trinityrnaseq.sourceforge.net/)(Grabherr et al., 2011). All the assembled transcripts were searched against the NCBI protein nonredundant (NR), String, and KEGG databases using BLASTX to identify the proteins that had the highest sequence similarity with the given transcripts to retrieve their function annotations and a typical cut-off E-values less than 1.0×10^{-5} was set. BLAST2GO (http://www. blast2go.com/ b2ghome)(Conesa et al., 2005) program was used to get GO annotations of unique assembled transcripts for describing biological processes, molecular functions and cellular components. Metabolic pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/)(Goto, 2000).

Differential expression analysis and Functional enrichment

To identify DEGs (differential expression genes) between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. RSEM (http:// deweylab. biostat.wisc.edu/rsem/) (B. Li & Dewey, 2011) was used to quantify gene and isoform abundances. R statistical package software EdgeR (Empirical analysis of Digital Gene Expression in R,http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) (Robinson, McCarthy, & Smyth, 2010) was utilized for differential expression analysis. In addition, functional-enrichment analysis include- ing GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P-value [?]0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/ Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do) (Xie et al., 2011).

Quantitative real-time PCR

We selected 19 (skin:10, brain:9) unigenes for sequencing results validation by qRT-PCR. The cDNA was transcribed from 2μg of total RNA using the Prime- ScriptTMII 1 st Strand cDNA Synthesis Kit in 20 μL of reaction mixture. qRT-PCR was performed with the Roche LightCycler® 96 Detection System (Applied Biosystems, Foster City, CA, USA) with Roche LightCycler®96 SYBR Green I Master (CWBIO, Beijing, China).The thermal profile for SYBR Green I RT-PCR was 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Gene ACTIN was used for normalization. The 2^{-CT} method was used to analyze the relative expression of these genes. All reactions were carried out in triplicate. Primers are listed in (Table .1.A; Table .1.B).

Statistical analysis

Statistical analyses were performed using variance (ANOVA) followed by Duncan's new multiple range tests with SPSS version 17.0. A significance level at $p < 0.05$ was applied. All of the experiments were repeated three times. Experiments with three independent biological replicates were performed for RNA-Seq (Sharma et al., 2019).

Results

Total RNA extraction and sequencing quality analysis

In this study, an average of 53339950 raw reads from NP_S and P_S (Table 2 A) were obtained and the average clean reads were 53001033. All the downstream analysis was based on the high-quality clean data. The error rates were 0.0231% and GC content were 49.29%. An average of 54229634 raw reads from NP_B and P_B (Table 2 B) were obtained and the average clean reads were 53595538. All the downstream analysis was based on the high-quality clean data. The error rates were 0.02% and GC content were 47.16%.

Defining differentially expressed genes (DEGs) at different degrees

Through the analysis of skin transcriptome sequencing data, 228 differentially expressed genes were obtained by comparing P_S with NP_S, including 126 up-regulated genes and 102 down-regulated genes (Fig 1 A; Fig

1 C). P_B compared with NP_B, 86 differentially expressed genes were obtained, including 71 up-regulated genes and 15 down-regulated genes (Fig 1 B; Fig 1 D) .

Functional annotation and classification of theDEGs

Public databases (NR, Swiss-Prot, KOG, COG, Pfam, GO, and KEGG) were used to annotate the DEGs. DEGs in skin Gene Ontology (GO) annotation showed that the obtained DEGs were classified into 10, 7 and 3 functional subcategories for biological process, cellular component and molecular function, respectively (Fig 2 A). Many genes were classified into more than one sub-category. The GO category of biological process showed a high percentage of cellular process, single-organism process. In terms of cellular component cell part and cell were high. With regard to molecular function, the top two GO terms were catalytic activity, binding. DEGs in brain Gene Ontology (GO) annotation showed that the obtained DEGs were classified into 9, 6 and 5 functional subcategories for biological process, cellular component and molecular function, respectively (Fig 2 B). Many genes were classified into more than one sub-category. The GO category of biological process showed a high percentage of cellular process. In terms of cellular component cell part, cell and organelle were high. With regard to molecular function, the top GO terms were binding.

DEGs in skin COG annotations showed that assigned to 24 COG categories (Fig 3 A). Among these functional categories, lipid transport and metabolism the largest group (4 DEGs) followed by amino acid transport and metabolism(3), translation, ribosomal structure and biogenesis (3), posttranslational modification, protein turnover, chaperones (3), general function prediction only(3). The smaller groups were nucleotide transport and metabolism (1), coenzyme transport (1) and transcription (1). DEGs in brain COG annotations showed that assigned to 24 COG categories (Fig 3 B). Among transcription (1), general function prediction only is the tow largest group (2 DEGs) followed by energy production and conversion (1), function unknown (1).

To further identify the biological pathways activated in skin, DEGs pathways were identified using the KEGG database (Fig 4 A). The KEGG pathway analysis showed 126 DEGs mapped into five major pathways: cellular process, environmental information processing, genetic information processing, metabolism, human diseases and organismal systems. Among the 37 biochemical pathways, the top three enriched pathways were signal transduction (17), immune system(15) and signaling molecules and interaction(12). Meanwhile, enriched pathways related to lipid metabolism and cellular community-eukaryotes were also found in the metabolism pathway. In addition, to further identify the biological pathways activated in brain, DEGs pathways were identified using the KEGG database (Fig 4 B). The KEGG pathway analysis showed 108 DEGs mapped into five major pathways: cellular process, environmental information processing, genetic information processing, metabolism, human diseases and organismal systems. Among the 29 biochemical pathways, the top three enriched pathways were signal transduction (12), nervous system(10) and cancers: overview (11). Meanwhile, enriched pathways related to signaling molecules and interaction.

DEGs in the skin and brain

Through KEGG enrichment analysis of differentially expressed genes in skin (Fig 5 A), 7 skin feeding related pathways were screened: Arachidonic acid metabolism pathway, Adhesion pathway, Apoptosis, Steroid biosynthesis, Tuberculosis, P53 signaling pathway, serotonergic synapse. The genes in the pathway were analyzed and ten significantly different genes associated with lactation were selected for validation. These DEGs included JUNB, MRC, DPP3, CASP3, PPID, ITGA11, ALOXE3, HBE, PTPRJ, GALE. Identically, through KEGG enrichment analysis of differentially expressed genes in brain (Fig 5 B), 20 lactation related pathways were screened: Estrogen signaling pathways, Inflammatory medlator regulation of TRP channels, non-small cell lung cancer, Vascular smooth muscle contraction, GnRH signaling pathway , TypeIIdiabetes mellitus, Neurotrophic signaling pathway, NOD-like receptor signaling pathway, Taste transduction, circadian rhythm, Jak-STAT signaling pathway, insulin resistance, Fc gamma R-mediated phagocytosis, Serotonergic synapses, Autophagy-animal, Small cell lung cancer, Pathways in cancer, AGE-RAGE signaling pathway in diabetic complications, Cytokin-cytokine receptor interaction. These DEGs included PRKCD, H1-5, EDNRB, LAPTM, FOXB, OTX2, NRIF2, SOX1 and HBE.

Validation and expression pattern analysis

To further validate the reliability of the sequencing data and the expression of DEGs, 19 DEGs were selected for expression analysis using qRT-PCR. The qRTPC- R results of genes were consistent with the RNA-Seq data (Fig 6 A; Fig 6 B), indicating that the transcriptomic data in the current study were reliable.

Discussion

In this study, 10 differentially expressed genes related to skin feeding of discus fish were selected, among which MRC, ITGA11 and PTPRJ were up-regulated in P_S period, JUNB, DPP3, CASP3, PPID, ALOXE3, HBE and GALE were down-regulated in P_S period. 9 differentially expressed genes were selected in brain of discus fish, among which EDNRB, LAPTM, FOXB, OTX2, NRIF2 and SOX1 were up-regulated in P_B period, HBE, PRKCD and H1-5 were down-regulated in P_B period.

One of arachidonic acid, the precursor of eicosanoids as paracrine hormones involving in various processes important in human health or disease, includes prostaglandins (PGs), leukotrienes and thrombox-anes. Arachidonic acid were the enriched fatty acid metabolisms (P. Krieg et al., 2002; Yoo et al., 2018). Arachidonate lipoxygenase3 (ALOXE3) gene is a member of the lipoxygenase family, which are catabolized by arachidonic acid-derived compounds and they are members of the epidermal subfamily of mammalian LOX with preferential expression in the skin and several other epithelial tissue (Peter Krieg et al., 2013). In this study, it was found that the expression of discus fish skin was different in NP_S and P_S periods, and decreased in P_S periods compared with NP_S periods. It encoded enzyme is a hydroperoxide isomerase that synthesizes a unique type of epoxy alcohol (8R-hydroxy-11R, 12R-epoxyeicosa-5Z, 9E, 14Z-trienoicacid) from 12R-hydroperoxyeicosatetraenoic acid (12R-HPETE). This epoxy alcohol can activate the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha), which is implicated in epidermal differentiation1 (Arias-Andres, Kettner, Miki, & Grossart, 2018; Yoo et al., 2018). Have study reported demonstrate the epidermal-type lipoxygenase. Aloxe3 is a potentially novel effector of the therapeutic fasting response (Sala et al., 2018). The discus fish will damage the skin tissue of the parents when pecking, and the content of Aloxe3 is reduced during the feeding period, which is consistent with Aloxe3 leads to a large amount of skin permeability and water loss leading to death after birth (Fournier et al., 2019).

PTPRJ a receptor-like protein tyrosine phosphatase (PTP) is expressed in several cell types. It is initially identified as a negative regulator of cell proliferation, and consistent with its increased expression in confluent epithelial and endothelial cells (Spring et al., 2012). Meanwhile, it has been suggested to contribute to cell-cell contact inhibition (Fournier, Dussault, Fusco, Rivard, & Royal, 2016). Many of its identified substrates are growth factor receptors, including VEGFR2, as well as ERK1/2 and cell adhesion proteins (Spring et al., 2012). PTPRJ dephosphorylates several growth factors and their receptors, negatively regulating cell proliferation and migration (Chabot, Spring, Gratton, Elchebly, & Royal, 2009). PTPRJ acts as an important regulator of Notch signaling and endothelial sprouting during retinal vascular development. Recent evidence suggests that the protein tyrosine phosphatase PTPRJ ACTS as a promoter of angiogenesis both in vivo and in vitro. There are abundant and dense capillaries on the skin surface of discus fish. The larvae absorb nutrients by biting the skin of the parent fish. We speculate that the nutrients absorbed by the larvae may be provided by the substances in capillaries in the skin of the parents of discus fish (Ghavami et al., 2009; Hashemi, Moazeni-Roodi, & Ghavami, 2019; Nagata, 2010; Spring, Lapointe, Caron, Langlois, & Royal, 2014). PTPRJ is the gene that we're going to focus on next.

Caspase 3 encoded by CASP3 gene located on the long arm of the chromosome. CASP3, an effector caspase, is activated by extrinsic and intrinsic apoptosis pathway and plays a key role in the execution phase of apoptosis (Nagata, 2010). Caspases, cysteine aspartate specific proteases, are involved in signaling and execution in apoptosis pathways. They can be generally divided into initiator and effector caspases based on their functions (Schorpp-Kistner, Wang, Angel, & Wagner, 1999). Apoptosis is a genetically programmed cell death process used to eliminate cells that are injured, infected, or have reached the end of their lifespan (Bender et al., 2008).The larva of discus fish pecked the parent's skin during lactation, which may damage the tissue cells of the skin and accelerate the rate of cell apoptosis. Therefore, it was speculated that Caspase 3 might be involved in the lactation process of discus fish.

JUNB is defined as a member of transcription factor family activator protein and a critical regulator and expression of inflammatory modulators in fibroblasts and T lymphocytes (Popov et al., 2011). The AP-1 transcription factor JUNB, which is encoded by the immediate early gene JUNB, mediates multiple biological pro-cesses including placentation, suppression of myeloid cell proliferation, and maintenance of bone and skin homeostasis. During lactation, the skin surface of discus fish is unstable, which may be related to the presence of JUNB and its decreased content. JUNB is a basic leucine zipper (bZIP) protein and forms an AP-1complex by dimerizing with other bZIP proteins, such as Fos or BATF family members (Puthiyaveetil, Kota, Chakkarayan, Chakkarayan, & Thodiyil, 2016; Sadri, Farhadi, & Nourmohamadi, 2019). It has been shown that JUNB is induced by ALK-NPM, participating the mTOR pathway (Yoo et al., 2018). In addition, JUNB acts as a regulator of vascular endothelial growth factor (VEGF) protein production and affects vessel proliferation and tissue angiogenesis. and acts as a VEGF production protein regulator and influences vessel proliferation and tissue angiogenesis (Popov et al., 2011). VEGF is a multifunctional cytokine that expresses in different situations and has a role in increasing vascular permeability and angiogenesis, thereby stimulating the proliferation and migration of endothelial cells (Bornstein, Agah, & Kyriakides, 2005). It's been reported that the lack of JUNB in different types of cells leads to very weak VEGF expression and postponed cell growth (Bornstein et al., 2005). During lactation, the expression of JUNB in larva of discus fish is decreased (Fig. 6.A), which may be caused by skin damage of discus fish.

The ITGA11 gene has been localized in bands q22.3 q23 on chromosome 15, and the gene encodes a mature protein with a large 1120-residue extracellular domain that contains an I-domain of 207 residues and is linked by a transmembrane domain to a short cytoplasmic domain of 24 amino acids (K. Lehnert et al., 1999). ITGA11 up-regulation has been shown to be dependent of TGFb-mediated signaling. The ITGA11 gene methylation status in the amnion may be valuable in explaining the mechanism of preterm birth (Klaus Lehnert et al., 1999). Generally, integrins and thrombospondins are known to play an important role for the regulation of cellular processes, such as cell adhesion, migration, and differentiation (Ji et al., 2011). ITGA11 gene expression is also increased through mid-gestation and decreased through the late stages of pregnancy (Yoo et al., 2018). This is consistent with increased expression of the ITGA11 gene during the breeding of discus (Fig 6 A). We speculate that the lactation process of angelfish is equivalent to the process of mammalian pregnancy, and the change of ITGA11 gene in P_S further confirms our speculation.

PRKCD, also known as protein kinase Cδ, was a protein kinase C isozyme that does not require calcium for its activity (Gschwendt, 1999). Activation of PPKCD was known to be associated with inhibition of cell cycle progression, suggesting that PRKCD had a negative effect on cell survival. Reported of PRKCD had been reported to be associated with apoptosis induced by various DNA damaging agents, including UV radiation, ionizing radiation, cisplatin, etoposide, cytosine arabinoside, mitomycin C and doxorubicin (Ke et al., 2013). During the two periods of discus fish, we found that P_B compared with NP_B, the content of PRKCD tended to decrease. It is possible that during lactation, the young fish will peck at the parent's skin, shortening the parent's skin cell cycle and resulting in a decrease in PRKCD content. PRKCD regulates membrane excitability by modulating different ion channels and pumps such as the calcium efflux regulator PMCA (plasma membrane calcium ATPase). In the skin, if the permeability barrier is disrupted by physical or mechanical damage, there is an increase on trans-epidermal water loss, followed by a decrease of the extracellular Ca^{2+} levels. The decrease in Ca^{2+} levels causes the opening of calcium channels allowing calcium influx to restore basal levels of Ca^{2+}. Accumulation of Ca^{2+} in the cell causes the activation of phospholipase C (PLCγ). The activated phospholipase cleaves phosphatidylinositol 4,5-biphosphate (PI (4,5) P2) generating DAG and inositol 1,4,5-triphosphate which in turn activate PRKCD. Thus, PRKCD is fundamental to maintain Ca^{2+}levels and the homeostatic balance in the skin (Malavez, Gonzalez-Mejia, & Doseff, 2009). During lactation, the discus fish pecked at the parents' skin, causing physical damage to the parents' skin and losing the balance of moisture and Ca^{2+} in the skin. This is consistent with the changes in PRKCD content caused by damage to the parents' skin during lactation of discus fish.

The process of feeding the larvae or pecking the parents of discus fish is a process of information exchange, and the external environment of the skin of discus fish also changes during the process of lactation and the discus brain has a neural regulation of environmental change, we found that the expression of the H1.5

gene changed during lactation. The H1.5-boundgenes function mainly in cell-cell communication and/or response to the environment. Linker histone H1.5 belongs to a family of proteins that organize eukaryotic DNA into a compact structure. The post-translational modifications induce conformational changes and allow the nuclear proteins to interact with chromatin which results in the regulation of transcription and gene expression during cell cycle (Ahmad et al., 2007). H1.5 forms blocks of chromatin binding in genic and intergenic regions in differentiated human cells from all germ layers but not in embryonic stem cells and it has a dynamic distribution during human cell differentiation and is required for maintenance of proper gene expression in differentiated cells (J.-Y. Li, Patterson, Mikkola, Lowry, & Kurdistani, 2012).

OTX2 gene encodes a member of the bicoid subfamily of homeodomain-containing transcription factors. The encoded protein acts as a transcription factor and plays a role in brain, craniofacial, and sensory organ development. The encoded protein also influences the proliferation and differentiation of dopaminergic neuronal progenitor cells during mitosis (https://www.gcbi.com.cn/gclib/html/dictSearch/T1RY- Mg). The milking behavior of discus fish is very similar to the feeding behavior of mammals, because the dopamine in mammals increases during reproduction, so it is possible that the dopamine in discus fish also increases during milking. So it's not surprising that increased OTX2 secretion was detected in the brain during the nurturing period.

HBE is downregulated when P_B compared with NP_B in discus fish brain (Fig. 6.B). However, it is the same in the skin as in the brain. HBE is a variant caused by a single point mutation at codon 26 of the b-globin gene, which is located on chromosome 11p15.5 (Ha, Martinson, Iwamoto, & Nishi, 2019). People possessing an HBE variant may also develop secondary disorders like jaun-dice, hepatosplenomegaly and growth retardation in their devel-opmental stages, which leads to the diagnosis of HBE (Fucharoen & Weatherall, 2012). Elizabeth Greene, Joshua Flees et al found the circulatory- and breast muscle-oxygen homeostasis is dysregulated [low oxygen and hemoglobin (HB) levels] in chickens with WB myopathy. Molecular analysis showed that blood HB subunit Mu (HBM), Zeta (HBZ), HBE and hephaestin (HEPH) expression were significantly down regulated (Fucharoen & Weatherall, 2012). Interestingly, this result was consistent with the downregulation of the relative expression of HBE in the lactation behavior of discus fish, suggesting that lactation behavior of discus fish is similar to that of mammals. HBE is the gene that we're going to focus on next.

This study has some limitations. First, the function of the genes screened by the transcriptome in the parental discus fish unclear. Second, the parental care process of discus fish is similar to that of mammals, but very different. Therefore, it is urgent to continue to explore the parental care process of discus fish.

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Data accessibility

The based Illumina PE library was constructed for 2×150bp sequencing using Illumina sequencing platform for transcriptome sequencing. Quality control (quality control) was conducted on the obtained sequencing data. Then the transcriptome data was analyzed by bioinformatics methods.

Author contributions

Y.L.W. and B.W analyzed and interpreted the data, drafted the manuscript and participated in designing the study. Z.Z.C and J.Z.G. acted as supervisor and advisor of the research and participated in analyzing the data.

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