Effects of gonadotropin-releasing hormone analog (GnRHa) immunization on the gonadal transcriptome and proteome of tilapia (*Oreochromis niloticus*)

Rui Wang
Guangxi Academy of Fishery Sciences
Luting Wen
Guangxi Academy of Fishery Sciences
Huawei Ma
Guangxi Academy of Fishery Sciences
Huizan Yang (yhzyang@163.com)
Guangxi Institution of Fishery Science
Min Lv
Guangxi Academy of Fishery Sciences
Zhong Chen
Guangxi Academy of Fishery Sciences
Xuesong Du
Guangxi Academy of Fishery Sciences
Lan Zeng
Guangxi Academy of Fishery Sciences
Yong Lin
Guangxi Academy of Fishery Sciences
Liming Huang
Guangxi Academy of Fishery Sciences
Tao Chen
Nanning University

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Abstract

Background: Gonadotropin releasing hormone (GnRH) plays an important role in the regulation of vertebrate reproduction. Studies have shown that immunization against GnRHa can induce sexually sterile tilapia. To explore the mechanism behind this, in this study, RNA-seq and data-independent acquisition (DIA) techniques were used to study the transcriptome and proteome of the gonad of tilapia immunized with GnRHa.

Results : 644 differentially expressed genes (80 upregulated and 564 downregulated) and 1150 differentially expressed proteins (351 upregulated and 799 downregulated) were identified. There were 209 genes with consistent differential expression patterns in the transcriptomic and proteomic analyses, of which 9 were upregulated and 200 downregulated, indicating that the gonad gene expression was inhibited by GnRHa immunization. The downregulated genes were particularly involved in the functions of single-organism process, binding, cellular process, metabolic process and catalytic activity, and associated with the pathways including ECM–receptor interaction, focal adhesion, cardiac muscle contraction and oxidative phosphorylation. The expression of six differentially expressed genes involved in the GnRH signaling pathway was all downregulated. In addition, several important functional genes related to gonadal development after GnRHa immunization were screened.

Conclusions: This study confirmed the expression of corresponding genes was affected by GnRHa on the gonad development in tilapia at the molecular level, and laid a foundation for elucidating the mechanism of GnRHa immunization.

Background

Gonadotropin releasing hormone (GnRH) is a neurohormone secreted by hypothalamic neurons, which plays important roles in the regulation of vertebrate reproduction. It is transported to the portal vein system through axons, flows into the anterior pituitary through blood vessels, binds to the GnRH receptor (GnRHR) on gonadotropin cells in the pituitary, and stimulates the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [1]. GnRH is a small-molecule polypeptide with weak immunogenicity, however, coupling with macromolecular protein carriers can improve its immunogenicity and induce immunized animals to produce GnRH antibodies [2]. GnRH antibody can neutralize endogenous GnRH, leading to infertility in male and female mammals [3]. The efficiency of GnRH purification is low due to the low content of endogenous GnRH, and synthetic gonadotropin releasing hormone analog (GnRHa) is often used as a clinical alternative. GnRHa not only retains the physiological functions of GnRH, but also exhibits a prolonged half-life and improved capacity to bind to GnRH receptors [4]. GnRHa antigen with good immunogenicity can be prepared by coupling GnRHa with bovine serum albumin (BSA) and emulsifying with Freund’s incomplete adjuvant [5]. GnRH immunization is considered to be an effective method to improve the growth performance of cultured animals, improve meat quality, control reproduction, and prevent undesired sexual behavior [6, 7]. The reproduction of fish is regulated by the release of gonadotropin from the pituitary induced by GnRH released from the hypothalamus [8]. Studies have shown that fish have two or three forms of GnRH [9], of which one or two forms can be found in the pituitary [10]. However, few reports have been published on the regulatory effect of GnRH immunization on the reproductive function of fish.

Tilapia is one of the most important freshwater culture varieties, exhibiting the advantages of rapid growth and strong stress resistance, which is an international fish that the Food and Agriculture Organization of the United Nations focuses on promoting aquaculture [11]. However, owing to its early sexual maturity and rapid breeding cycle, the reproductive behavior of tilapia in mixed-sex culture consumes a lot of energy, which interferes with growth, and at the same time leads to very different qualities of harvested tilapia, a low commodity rate, and a high feed conversion ratio, greatly reducing the product quality and associated economic benefits [12]. As such, it is important to obtain sterile individuals to eliminate the adverse effects of sexual maturity and reproductive behavior of tilapia. Our previous studies showed that the level of GnRH antibody in the GnRHa immunized group was significantly higher than that in the negative control group [13]. To further investigate the mechanism behind the effects of GnRHa immunization in tilapia, the transcriptome and proteome of gonad of tilapia after GnRHa immunization were analyzed, and the genes with functions that are particularly important for gonad development of tilapia were screened. The results confirmed the expression of corresponding genes was affected by GnRHa on gonad development at the molecular level, and laid a foundation for elucidating the mechanism of GnRHa immunization.

Results

RNA-seq

The quality control results of the original data of RNA-seq showed that a total of 375.2 million clean reads were obtained from the eight samples after preliminary filtration, and the average number of clean reads per sample was 46.9 million (ranging from 35.9 to 59.5 million). Overall, 98.45–98.98% high-quality clean reads were obtained after further filtration of the clean reads, and the filtered reads included 0.31–0.36% reads containing an adapter, 0.71–1.21% low-quality reads (containing more than 50% bases with Qvalue ≤ 20), and 53–148 reads containing all A, no reads containing more than 10% N. After quality control, the data were considered to be of sufficient quality to be used for subsequent analysis. Owing to the sample quality and species, the efficiency of removing ribosomal RNA might not be stable, and the contamination of ribosomal RNA would affect the subsequent analysis. Therefore, the read alignment tool was applied to align high-quality clean reads to the ribosome of the species (mismatch number: 0), and the aligned reads were removed; the remaining data were used for subsequent analysis. The alignment results showed that the proportion of unmapped reads obtained from the samples was 98.35–99.81%. The high-quality clean reads without ribosomal RNA were aligned to the reference genome (GCF_001858045.2) of tilapia using TopHat2 (2.1.1), the results of which are shown in Table 1. There were 10.95–13.36% unmapped reads, 85.54–88.00% uniquely mapped reads, and 1.05–1.78% multiple mapped reads, resulting in an overall mapping rate of 86.64–89.05%.

Table 1

Genome comparison statistics

Sample	Total Reads	Unmapped Reads	Unique Mapped Reads	Multiple Mapped reads	Mapping Ratio
CK-1	36590112	4886623 (13.36%)	31300681 (85.54%)	402808 (1.10%)	86.64%
CK-2	47379594	5866217 (12.38%)	40972731 (86.48%)	540646 (1.14%)	87.62%
CK-3	38326808	4471997 (11.67%)	33337199 (86.98%)	517612 (1.35%)	88.33%
CK-4	35287440	3998243 (11.33%)	30883381 (87.52%)	405816 (1.15%)	88.67%
T1-1	54158908	6050754 (11.17%)	47145836 (87.05%)	962318 (1.78%)	88.83%
T1-2	45581802	5197612 (11.40%)	39648236 (86.98%)	735954 (1.61%)	88.60%
T1-3	58683152	6760559 (11.52%)	51057149 (87.00%)	865444 (1.47%)	88.48%
T1-4	51930932	5684817 (10.95%)	45700263 (88.00%)	545852 (1.05%)	89.05%

Gene statistics

The alignment results of the protein-coding genes of each sample are shown in Table 2. The total number of genes from the reference genome was 29,550, and 28,209 known genes (95.46%) and 1796 unknown genes were detected in the sequencing results. The total number of genes in the immunized group (T1) was 29,370, including 27,603 known genes (93.41%) and 1767 unknown ones, while the total number of genes in the control group (CK) was 28,363, including 26,810 known genes (92.73%) and 1553 unknown ones.

Table 2
Numbers of detected genes in each sample

Sample Name	Known Gene Num	New Gene Num	All Gene Num
CK-1	24056 (81.41%)	1326	25382
CK-2	24460 (82.77%)	1322	25782
CK-3	24245 (82.05%)	1303	25548
CK-4	24031 (81.32%)	1344	25375
T1-1	22592 (76.45%)	1332	23924
T1-2	22712 (76.86%)	1233	23945
T1-3	23968 (81.11%)	1333	25301
T1-4	25996 (87.97%)	1139	27135

Identification of DEGs

In this study, edgeR software was used to analyze the difference of gene expression between the groups, and FDR and $\log_2$FC were used to screen the DEGs. A total of 644 DEGs were obtained. As shown in Fig. 1, 80 upregulated genes and 564 downregulated genes were identified in the immunized group (T1), compared with the control group (CK).

GO and KEGG analyses of DEGs

The DEGs were annotated by GO, and then the upregulated and downregulated genes was conducted by the GO term classification. The results (Fig. 2) showed that the upregulated genes were mainly involved in catalytic activity (13 genes) and metabolic process (11 genes), while the downregulated genes were mainly related to single-organism process (116 genes), binding (113 genes), cellular process (103 genes), metabolic process (83 genes), and catalytic activity (73 genes).

Functional enrichment analysis of the DEGs revealed the 20 most enriched pathways, as shown in Fig. 3. The RichFactor indicates the ratio of the number of differentially expressed transcripts enriched in the pathway relative to the total number of transcripts enriched in the pathway; a higher RichFactor indicates a higher degree of enrichment. QValue is the P value after correction for multiple testing, which ranges from 0 to 1; a smaller QValue indicates more significant enrichment. The 20 pathways with the smallest Qvalue are shown in Fig. 3. The 20 enriched pathways included ECM–receptor interaction (24 related genes), focal adhesion (30 related genes), cardiac muscle contraction (15 related genes), oxidative phosphorylation (10 related genes), regulation of actin cytoskeleton (16 related genes), adrenergic signaling in cardiomyocytes (13 related genes), steroid hormone biosynthesis (5 related genes), phagosome (14 related genes), PPAR signaling pathway (5 related genes), GnRH signaling pathway (6 related genes), glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate (2 related genes), α-linolenic acid metabolism (2 related genes), ether lipid metabolism (3 related genes), endocytosis (14 related genes), cytokine–cytokine receptor interaction (12 related genes), cell adhesion molecules (CAMs; 13 related genes), arginine and proline metabolism (3 related

genes), β-alanine metabolism (2 related genes), gap junction (5 related genes), and arachidonic acid metabolism (4 related genes).

The six GnRH signaling pathway-related genes were epidermal growth factor receptor, transcript variant X1 (EGFR), matrix metalloproteinase-14 (MT1), matrix metallopeptidase 2 (MMP2), transcription factor AP-1 (c-Jun), voltage-dependent L-type calcium channel subunit alpha-1D-like (CACN), and cytosolic phospholipase A2 gamma-like (PLA2); their locations in the GnRH signaling pathway are shown in Fig. 4.

Identification of DEPs

Quality control of the original mass spectrometry data was carried out using QuiC software (Biognosys), and the similarity of quality control indexes between samples was determined. Then, a library was established based on the data obtained by DDA mode using Pulsar software, and was used for the quantification of subsequent DIA data. The identification standard of proteins was as follows: peptide precursor threshold 1.0% FDR and protein threshold 1.0% FDR. There were a total of 70,334 peptide precursors, 56,726 peptides, 5,700 protein groups, and 10,468 proteins. The local normalization method in Pulsar software was used to normalize the quantitative results of peptide DIA in all samples and eliminate the systematic errors. The expression of a protein was the median of the relative expression of all unique peptides of the protein. The annotation of identified proteins included GO annotation, pathway annotation, and COG/KOG annotation. The screening threshold of DEPs was more than 1.5 times the absolute value of the relative quantitative values and Student's t-test Qvalue < 0.05. A total of 1,150 DEPs were identified, of which 351 were upregulated and 799 were downregulated in T1, compared with CK (Fig. 1).

GO and KEGG analyses of DEPs

The DEPs were annotated to GO terms, which were then classified according to the up- or downregulation (Fig. 5). The results showed that the DEPs were mainly enriched in metabolic process (53 upregulated and 181 downregulated), single-organism process (35 upregulated and 149 downregulated), and cellular process (53 upregulated and 153 downregulated). The upregulated and downregulated proteins within the category of molecular function were mainly related to catalytic activity (39 upregulated and 134 downregulated) and binding (56 upregulated and 126 downregulated), while the upregulated and downregulated genes within the category of cellular component were mainly related to organelle (16 upregulated and 68 downregulated), cell (34 upregulated and 91 downregulated), and cell part (34 upregulated and 91 downregulated).

According to the pathway enrichment analysis of DEPs, the 20 most enriched pathways were determined, as shown in Fig. 6. These included 33 involved in protein processing in endoplasmic reticulum, 11 in protein export, 122 in metabolic pathways, 16 in fatty acid degradation, 13 in PPAR signaling pathway, 5 in fatty acid biosynthesis, 7 in N-glycan biosynthesis, 13 in peroxisome, 11 in fatty acid metabolism, 23 in oxidative phosphorylation, 4 in dorsal–ventral axis formation, 16 in lysosome, 9 in arginine and proline metabolism, 4 in other glycan degradation, 7 in glycerolipid metabolism, 3 in glycosaminoglycan degradation, 10 in ECM–receptor interaction, 9 in amino sugar and nucleotide sugar metabolism, 9 in myocardial contraction, and 7 in propionate metabolism.

Correlation analysis of transcriptome and proteome in tilapia

The correlations of the transcriptome and proteome of tilapia in the immunized group and the control group were analyzed, and functional enrichment analysis of the related gene sets was carried out. The results are shown in Fig. 7. The findings for a total of 209 mRNAs were consistent with the differential expression pattern of the corresponding proteins, of which 9 were upregulated and 200 were downregulated. Significance analysis showed that the findings of 48 mRNAs were consistent with the differential expression patterns of the corresponding proteins, of which 1 was upregulated and 47 were downregulated. The detailed information of the genes with a consistent pattern are shown in Table 3.

Table 3

Genes with a consistent pattern of differential expression between transcriptome and proteome

No.	mRNA[a]	Protein[a]	Description	Pathway
upregulated				
1	3.12	1.60	sterile alpha motif domain-containing protein 3-like, transcript variant X1	-
downregulated				
1	-11.18	-5.66	zona pellucida sperm-binding protein 3	-
2	-10.81	-1.37	apolipoprotein A-I	-
3	-9.12	-1.47	uncharacterized LOC109201766	-
4	-6.57	-0.88	NADH dehydrogenase subunit 4	ko01100//Metabolic pathways;ko00190//Oxidative phosphorylation
5	-5.85	-0.62	actin, cytoplasmic 1	ko04145//Phagosome;ko04810//Regulation of actin cytoskeleton;ko04510//Focal adhesion;ko04530//Tight junction;ko04520//Adherens junction
6	-5.45	-1.00	betaine–homocysteine S-methyltransferase 1	ko01100//Metabolic pathways;ko00270//Cysteine and methionine metabolism;ko00260//Glycine, serine and threonine metabolism
7	-5.29	-0.88	apolipoprotein A-I	ko03320//PPAR signaling pathway
8	-4.90	-1.28	Ladderlectin, transcript variant X1	-
9	-4.66	-1.35	Actin, alpha, cardiac muscle 1	ko04261//Adrenergic signaling in cardiomyocytes;ko04260//Cardiac muscle contraction
10	-4.58	-1.45	type-4 ice-structuring protein	-
11	-4.36	-2.50	proteasome subunit beta type-9	ko03050//Proteasome
12	-4.29	-0.60	integrin alpha-X	ko04810//Regulation of actin cytoskeleton
13	-4.19	-1.14	immunoglobulin lambda-1 light chain-like	-

a: Log 2-fold change of differentially expression

No.	mRNA[a]	Protein[a]	Description	Pathway
14	-3.94	-2.05	seminal plasma glycoprotein 120	-
15	-3.74	-1.00	NADH dehydrogenase subunit 2	ko01100//Metabolic pathways;ko00190//Oxidative phosphorylation
16	-3.58	-2.40	retbindin	ko04144//Endocytosis
17	-3.53	-3.49	extracellular matrix protein 1	-
18	-2.87	-0.64	prolyl 4-hydroxylase subunit alpha 1	ko01100//Metabolic pathways;ko00330//Arginine and proline metabolism
19	-2.85	-1.67	linkage group 7 C11orf96 homolog	-
20	-2.79	-2.61	hydroxysteroid 17-beta dehydrogenase 1	ko01100//Metabolic pathways;ko00140//Steroid hormone biosynthesis
21	-2.76	-0.77	15-hydroxyprostaglandin dehydrogenase [NAD(+)], transcript variant X2	-
22	-2.35	-1.10	extracellular matrix protein 1	-
23	-2.29	-1.04	progesterone receptor	ko04114//Oocyte meiosis;ko04914//Progesterone-mediated oocyte maturation
24	-2.19	-0.85	NADH-cytochrome b5 reductase 2	ko00520//Amino sugar and nucleotide sugar metabolism
25	-2.13	-1.26	gap junction Cx32.7 protein	ko04540//Gap junction
26	-2.12	-0.71	Elastin, transcript variant X1	-
27	-2.10	-0.67	glucose transporter	ko04920//Adipocytokine signaling pathway
28	-2.03	-0.95	progesterone receptor membrane component 1	-
29	-2.03	-1.46	synuclein gamma, transcript variant X2	-
30	-1.94	-0.68	carboxypeptidase N subunit 1	-

a: Log 2-fold change of differentially expression

No.	mRNA[a]	Protein[a]	Description	Pathway
31	-1.88	-0.72	procollagen C-endopeptidase enhancer 2	-
32	-1.84	-1.42	switch-associated protein 70	-
33	-1.73	-2.83	complement receptor type 1, transcript variant X1	-
34	-1.64	-0.64	collagen alpha-4(IV) chain	ko04510//Focal adhesion;ko04512//ECM-receptor interaction
35	-1.62	-1.70	selenoprotein P	-
36	-1.59	-1.24	thymosin beta 4 X-linked	ko04810//Regulation of actin cytoskeleton
37	-1.57	-1.50	thrombospondin type-1 domain-containing protein 4	-
38	-1.55	-1.07	beta-2-microglobulin	-
39	-1.50	-1.71	thrombospondin-1b, transcript variant X1	ko04145//Phagosome;ko04510//Focal adhesion;ko04512//ECM-receptor interaction;ko04350//TGF-beta signaling pathway;ko04115//p53 signaling pathway
40	-1.48	-1.37	peptidyl-prolyl cis-trans isomerase FKBP10	-
41	-1.18	-1.11	laminin subunit beta-1, transcript variant X1	ko04510//Focal adhesion;ko04512//ECM-receptor interaction
42	-1.14	-0.73	integrin subunit alpha V, transcript variant X1	ko04514//Cell adhesion molecules (CAMs);ko04145//Phagosome;ko04810//Regulation of actin cytoskeleton;ko04510//Focal adhesion;ko04512//ECM-receptor interaction
43	-1.12	-1.42	secreted protein acidic and cysteine rich	-
44	-1.11	-1.44	serpin family E member, transcript variant X1	-
45	-1.03	-1.32	Cystatin, transcript variant X1	-
46	-1.01	-2.71	golgin A4, transcript variant X1	-

a: Log 2-fold change of differentially expression

No.	mRNA[a]	Protein[a]	Description	Pathway
47	-1.01	-1.36	ribosomal protein L39	ko03010//Ribosome
a: Log 2-fold change of differentially expression				

Validation of RNA-seq results by quantitative real-time reverse transcription-PCR (qRT-PCR)

To further validate the results from the RNA sequencing data, six genes were selected for qRT-PCR analysis. Of the six selected genes, all showed similar expression patterns in the qRT-PCR analysis as observed from RNA-seq data (Fig. 8). The statistical analysis also showed very good correlation (r = 0.997) between the two types of analysis.

Discussion

In addition to genetic factors, external environmental factors also play important roles in the sex determination and differentiation of fish. Therefore, fish can be useful subjects to study sexual differentiation. With the continuous progress and development of transcriptomic and proteomic technologies, transcriptomics and proteomics have been widely used in the study of reproductive and sexual differentiation-related mechanisms in fish, including tilapia, especially in the identification of DEGs, the screening of functional genes, and the discovery and verification of important regulatory pathways [14–17]. After treatment with progesterone receptor inhibitor (RU486), 7148 genes were differentially expressed in gonad of female tilapia; the results revealed that fshr and lhr were significantly downregulated and ars was significantly upregulated after RU486 treatment, which might account for the masculinization and infertility of female fish [18]. Transcriptomic analysis of the gonads of female and male tilapia at different developmental stages revealed that estrogen may play an important role in female sex determination and maintenance of phenotypic sex, which lays the foundation for future studies into the molecular mechanisms of sex determination and maintenance of phenotypic sex in non-model teleosts [19]. Analysis of the transcriptome of the gonads of control female, high-temperature-treated female, and high-temperature-induced neomale tilapia identified a number of genes that may be involved in GSD + TE (genotypic sex determination + temperature effects), which should be useful for investigating the molecular mechanisms of GSD + TE in fish [20]. Study of the gonadal proteome in fish during sex reversal or gonadal differentiation, and the screening of important functional proteins related to reverse acquisition and gonadal differentiation are of great significance for the study of vertebrate sex determination and differentiation [21, 22]. GnRH immunization of mammals, such as mice, sheep, cattle, and pigs, can reduce the levels of FSH and LH, and inhibit the development of the gonads, achieving artificial intervention in animal sex differentiation [12]. Intervention in fish sex differentiation by GnRH immunization, investigation of the mechanism of action of GnRH in regulating sex differentiation, and screening of sex determination factors are of great significance for uncovering the sex-differentiation and sex-controlling mechanisms. However, no transcriptomic or proteomic studies of tilapia gonad after GnRH immunization have been reported.

In this study, the gonadal transcripts of tilapia immunized with GnRHa were sequenced, and an average of 88.28% clean reads were mapped to the reference genome. Sun et al. sequenced the gonadal transcripts and

mapped an average of 80.09% clean reads to the reference genome [20]. The reference genome of tilapia used by Sun et al. was Orenil1.1 (GCA_000188235.2) [20], while the reference genome used in this study was O_niloticus_UMD_NMBU (GCA_001858045.3). Orenil1.1 is a genome assembled by next-generation sequencing (Illumina), with a full length of 927,679,487 bp, while O_niloticus_UMD_NMBU was assembled by third-generation sequencing (PacBio), with a full length of 1,005,681,550 bp. Combined with other genome parameters, the integrity of the O_niloticus_UMD_NMBU genome is better than that of the Orenil1.1 genome, resulting in better alignment in this study. The failure in aligning 11.72% clean reads to the reference genome in this study might have been due to factors such as wrongly sequenced reads remaining after quality control, interference of alternatively spliced genes, contamination by other genomes, and poor integrity and assembly of the reference genome.

In this study, 644 DEGs were identified by transcriptomic analysis, including 80 upregulated genes and 564 downregulated ones. Moreover, 1150 DEPs were identified by proteomic analysis, including 351 upregulated proteins and 799 downregulated ones. Overall, 209 genes showed consistent differential expression patterns at mRNA and protein levels, of which 9 were upregulated and 200 were downregulated. The results of the three kinds of analysis showed that the expression of gonadal genes was inhibited by GnRHa immunization in tilapia, and the functions of downregulated genes were mainly focused on single-organism process, binding, cellular process, metabolic process, and catalytic activity, while the main involved pathways include ECM–receptor interaction, focal adhesion, cardiac muscle contraction, and oxidative phosphorylation. It is notable that six DEGs are involved in the GnRH signaling pathway, and the expression of these six genes is downregulated, indicating that GnRHa immunization can inhibit the expression of genes related to the gonadotropin releasing hormone signal transduction pathway.

Forty-seven downregulated genes were obtained by combined analysis of the transcriptomic and proteomic data, among which the differential expression of zona pellucida sperm-binding protein 3 (ZPBP3), apolipoprotein A-I (apo A-I), and cytoplasmic 1 was most significant. Zona pellucida sperm binding protein (ZPBP) is closely related to sperm motility, capacitation, acrosome reaction, and sperm–egg binding. Studies have shown that ZPBP1 and ZPBP2 were mainly expressed in testicular tissue of mammals, while ZPBP3 was found in tilapia [23, 24]. In this study, it was found that ZPBP3 could be expressed in the gonad of female tilapia, which may be related to the specific sex differentiation mechanism of fish. Meanwhile, the expression of ZPBP3 in gonad of tilapia was significantly downregulated after GnRHa immunization, indicating that ZPBP3 plays an important role in the gonad development of tilapia. Apo A-I is the major component of high-density lipoprotein, which plays an important role in reverse cholesterol transport and lipid metabolism, and is also a very important innate immune molecule, providing a platform for the assembly of several immune complexes [25, 26]. Previous studies showed that apo A-I was present in multiple tissues of Branchiostoma belcheri, and the expression of apo A-I mRNA was highest in the gonads; the expression of apo A-I mRNA increased significantly in the process of infection, suggesting that apo A-I may be involved in immune stress response and play an important role in the innate defense immune system of Amphioxus [27]. The apo A-I expression profile in Monopterus albus immunized with Aeromonas hydrophila showed that infection could downregulate the expression of apo A-I in the small intestine, spleen, and liver, indicating that apo A-I might be involved in the innate immune system of this species [28]. In this study, the expression of apo A-I in gonad of tilapia immunized with GnRHa was significantly downregulated, which also indicated that apo A-I plays an

important role in the immune response of tilapia. Actin is one of the most highly conserved proteins in eukaryotic cells. It is mainly present in the cytoplasm, is the main component of cytoskeleton microfilaments, and is necessary for a variety of cell functions, such as the division, movement, and growth of cells [29]. Actin (cytoplasmic 1) can be expressed in several tissues of tilapia, but its expression level in gonad is much higher than that in other tissues, indicating that this gene may play an important role in gonad development and gonad functions of tilapia [24]. In this study, the expression of actin (cytoplasmic 1) in gonad of tilapia immunized with GnRHa was significantly downregulated, which indicated that GnRHa immunization could inhibit the expression of important genes involved in gonad development and provide a reference for revealing the mechanism of GnRH immunization.

Conclusions

To elucidate the mechanism behind the immunosuppressive effect of GnRHa on gonad development in tilapia, this study analyzed the transcriptome and proteome of tilapia gonad after GnRHa immunization. The results showed that, compared with that in the control group, the gonad gene expression after GnRHa immunization was significantly different, and the number of downregulated genes was significantly higher than that of the upregulated genes. Six differentially expressed genes involved in the GnRH signaling pathway were screened, all of which were downregulated, indicating that GnRHa immunization inhibited the expression of genes related to the gonadotropin releasing hormone signal transduction pathway in tilapia. In addition, several important functional genes related to GnRHa immunity were screened, which laid a foundation for revealing the mechanism behind the effect of GnRH on gonad development in tilapia. Further studies on the expression patterns of screened GnRHa immune-related genes and the verification of gene functions are currently underway.

Methods

Fish and GnRHa immunization

All female tilapia (Oreochromis niloticus) with average weight of 30.22 ± 1.80 g were provided by the National Tilapia Seed Farm (Nanning, Guangxi, China). According to the amino acid sequence of tilapia GnRH in Uniprot database (entry name: Q76FQ2), we had designed and synthesized tilapia GnRHa (Pyr-His-Trp-Ser-Tyr--Leu-Arg-Pro-NHEt). The GnRHa antigen was prepared according to the method we reported before [13]. The tilapia were randomly divided into two groups. One group (the GnRHa-immunized group; T1) was immunized by intraperitoneal injection with 0.5 mL/fish of GnRHa Freund's complete adjuvant antigen (200 μg/mL), followed by the same dose of GnRHa Freund's incomplete adjuvant antigen after 14 days. The other group (the control group; CK) was injected with the same volume of PBS emulsion. The samples were collected 10 weeks after the first immunization. Four replications were included in each group with 60 tilapia per replication, which were named T1-1, T1-2, T1-3, and T1-4 and CK-1, CK-2, CK-3, and CK-4. During the study, the water temperature of tilapia culture was kept at 27.5 ± 1.5 °C, and the water was aerated continuously for 24 h. The water was refreshed every 2 days and the fish were provided with feed equivalent to 3% of their body weight daily. The basic fish feed comprising soybean meal, fish meal, and flour was used as feed (purchased from Nanning Tongwei Feed Company; floating fish feed).

Sampling and gender identification

Feeding was stopped 24 h prior to sampling. The sex of tilapia was judged according to the external genitalia. The fishes were anesthetized with MS222. Then, the complete gonad was isolated and washed with PBS. For each repetition, the gonads of three tilapia were mixed, put into an RNase-free cryotube, and stored at − 80 °C after freezing in liquid nitrogen.

RNA extraction, RNA-seq library construction, and Illumina deep sequencing

The total RNA of tissues was extracted using Trizol reagent (Invitrogen), in accordance with the manufacturer's instructions. Then, genomic DNA was removed from the RNA sample using DNase. Two microliters of total RNA was separated by 1% agarose gel electrophoresis to preliminarily determine the integrity and purity of RNA samples. Then, the concentration, quality, and integrity of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples with 260/280 nm absorbance ratios of approximately 2.0 and RNA integrity numbers (RIN) > 7.0 were processed for RNA-seq.

The mRNA was enriched using oligo(dT) beads, and the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse-transcribed into cDNA with random primers. Second-strand cDNA was synthesized using DNA polymerase I, RNase H, dNTP, and buffer. Then, the cDNA fragments were purified with QiaQuick PCR extraction kit, end-repaired, poly(A)-added, and ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, PCR-amplified, and sequenced using Illumina HiSeq™ 4000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Bioinformatic analysis of RNA-seq data

To acquire clean reads, the raw reads were filtered by removing reads containing adapters, reads containing more than 10% unknown nucleotides (N), and low-quality sequences [reads containing more than 50% low-quality (Qvalue ≤ 10) bases]. To avoid interference of ribosomal RNA, we mapped the clean reads to the ribosome database using bowtie [30] and removed the matched reads from the ribosome database. We mapped the remaining reads to the genome sequence of tilapia (GCF_001858045.2) using TopHat2 [31]. The sequencing data were deposited in the NCBI Sequence Read Archive under accession numbers SRR9937067–SRR9937074. The aligned reads were assembled into transcripts using cufflinks, and the assembled transcripts were merged using cuffmerge [32]. The abundance of gene transcripts was calculated via FPKM (fragments per kilobase of transcript per million mapped reads) [33], and genes with mean abundance of > 0 FPKM in any one of these samples were regarded as being expressed.

The raw read counts were used for differential expression analysis using edgeR [34]. Genes with fold change of FDR < 0.05, $|\log_2 FC| > 1$, and $p < 0.05$ were considered to be differentially expressed. In this study, the differentially expressed genes (DEGs) between the immunized group (T1) and the control group (CK) were analyzed. After obtaining the DEGs, Gene Ontology (GO) classification was performed on them using the GOseq R packages based on a Wallenius noncentral hypergeometric distribution [35], which can adjust for

gene length bias in DEGs. The statistically significant enrichment of DEGs in KEGG pathways was tested using KOBAS software [36].

Protein extraction, protein digestion, and separation high pH reverse phase

Total proteins were extracted from the same samples as those for RNA-seq using the cold acetone method [37]. Protein quality was examined using SDS-PAGE. The Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used to determine the concentration of protein. A total of 50 μg of protein extracted from tilapia gonad was suspended in 50 μL of solution, reduced by adding 1 μL of 1 M dithiothreitol at 55 °C for 1 h, and alkylated by adding 5 μL of 20 mM iodoacetamide in the dark at 37 °C for 1 h. Then, the sample was precipitated using 300 μL of prechilled acetone at − 20 °C overnight. The precipitate was washed twice with cold acetone and then resuspended in 50 mM ammonium bicarbonate. Finally, the proteins were digested with sequence-grade modified trypsin (Promega) at a substrate/enzyme ratio of 50:1 (w/w) at 37 °C for 16 h.

Data-dependent acquisition (DDA) mass spectrometry

The peptide mixture of the eight samples was redissolved in buffer A (20 mM ammonium formate in water, pH 10.0, adjusted with ammonium hydroxide) and then fractionated by high pH separation using the Ultimate 3000 system (ThermoFisher Scientific) connected to a reverse-phase column (XBridge C18 column, 4.6 mm × 250 mm, 5 μm; Waters Corporation). High pH separation was performed using a linear gradient, starting from 5% B (20 mM ammonium formate in 80% ACN, pH 10.0, adjusted with ammonium hydroxide) to 45% B in 40 min. The column was re-equilibrated at the initial conditions for 15 min. The column flow rate was maintained at 1 mL/min and the column temperature was maintained at 30 °C. Ten fractions were collected; each fraction was dried in a vacuum concentrator for the next step.

The ten fractions were redissolved in 30 μL of C (0.1% formic acid in water) and analyzed by online nanospray LC-MS/MS on an Orbitrap Fusion Lumos (Thermo Fisher Scientific) coupled to a Nano ACQUITY UPLC system (Waters Corporation). A 10 μL peptide sample was loaded onto the trap column (Acclaim PepMap C18, 100 μm × 2 cm; Thermo Fisher Scientific) with a flow rate of 300 nL/min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm × 15 cm) with a set gradient, from 5% D (0.1% formic acid in ACN) to 8% D in 3 min; from 8% D to 22% D, 3–82 min; from 22% D to 35% D, 82–106 min; from 35% D to 90% D, 106–118 min; maintained at 90% D for 5 min; from 90% D to 3% D in 0.1 min; and then maintained at 3% D to 120 min. The column flow rate was maintained at 500 nL/min with a column temperature of 40 °C. An electrospray voltage of 2.1 kV versus the inlet of the mass spectrometer was used.

The mass spectrometer was run under data-dependent acquisition mode, and automatically switched between MS and MS/MS modes. The parameters were as follows: (1) MS: scan range (m/z) = 350–1200; resolution = 60,000; AGC target = 400,000; maximum injection time = 50 ms; included charge states = 2–6; filter dynamic exclusion: exclusion duration = 30 s; and (2) HCD-MS/MS: resolution = 30,000; AGC target = 500,000; maximum injection time = 64 ms; collision energy = 35%; and stepped CE = 5%.

Quantification of proteins using data-independent acquisition (DIA) mass spectrometry

The peptides of eight samples were redissolved in 30 μL of C and analyzed by HPLC as mentioned above. The mass spectrometer was run under DIA mode, and automatically switched between MS and MS/MS modes. The parameters were as follows: (1) MS: scan range (m/z) = 350–1200; resolution = 120,000; AGC target = 500,000; maximum injection time = 60 ms; and (2) HCD-MS/MS: resolution = 50,000; AGC target = 1,000,000; maximum injection time = 100 ms; collision energy = 35%; and stepped CE = 5%.

Mass spectrometric data analysis

Raw data of DDA were processed and analyzed by Spectronaut Pulsar 11.0 (Biognosys AG). Pulsar was set up to search the database of O. niloticus assuming trypsin as the digestion enzyme. Carbamidomethylation was specified as the fixed modification. Oxidation was specified as the variable modification. Raw data of DIA were processed and analyzed by Spectronaut Pulsar 11.0 (Biognosys AG) with the default parameters. After Student's t-test, differentially expressed proteins (DEPs) were filtered if their Qvalue was < 0.05 and absolute AVG $\log_2$ ratio was > 0.58.

Protein functional annotation and enrichment analysis

Proteins were annotated against the GO, KEGG, and COG/KOG databases to obtain their functions. Significant GO functions and pathways were examined among the DEPs with Qvalue ≤ 0.05.

Validation of DEGs by qRT-PCR

To validate the DEGs obtained by RNA sequencing, qRT-PCR was carried out with the total RNA used for RNA sequencing to examine the expression of 6 DEGs (2 upregulated unigenes and 4 downregulated unigenes). In the qRT-PCR analysis, the β-actin gene was amplified as an endogenous control. All samples were tested in triplicate. Specific primers were designed by the Primer Express 3.0 Software (Applied Biosystems), and are listed in Table 4. The relative changes of gene expression were calculated by $2^{-\Delta\Delta Ct}$ methods [38]. The value of log 2-fold change was used for graphing.

Table 4
Primer sequences used for quantitative real-time reverse transcription-PCR.

Transcript ID	Transcript name	Forward Primer	Reverse Primer
ncbi_109194882	uncharacterized	GCCATCACTGTTCCCATCCA	CACCCTCCGTAACCTCGTCT
ncbi_109195722	uncharacterized	GCCATCACTGTTCCCATCCA	CACCCTCCGTAACCTCGTCT
ncbi_100695709	EGFR	ATGCTGTGGACGCTGATGAA	GATGCTGCTGTTGAGGCTTG
ncbi_100695964	MT1	GGATAAGTGGTTCTGGCGGG	GGTGTAGTAGAGAGCAGCGT
ncbi_100700606	MMP2	GCTACCCGAAAAGGTTGTCC	TGATTTCACCCAACTTCACGAT
ncbi_100703685	c-Jun	ACCTCAGCCCCCACTATGTC	GCTCGGAGTTCTGTGACTTTAGG
–	actb	CTTCAACACCCCCGCCA	ATGTCACGCACGATTTCCCT

Abbreviations

GnRHa: gonadotropin-releasing hormone analog; DIA: data-independent acquisition; FSH: follicle-stimulating hormone; LH: luteinizing hormone; FDR: false discovery rate; FC: fold change; T1: immunized group; CK: control group; DEGs: differentially expressed genes; DEPs: differentially expressed proteins; DDA: data-dependent acquisition; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; qRT-PCR: quantitative real-time reverse transcription-PCR; FPKM: fragments per kilobase of transcript per million mapped reads.

Declarations

Ethics approval and consent to participate

All experiments were conducted under the Guidelines of the Laboratory Animals Use and Care of China. This study was approved by the Committee on the Ethics of Animal Experiments of Guangxi Academy of Fishery Sciences.

Consent to publish

Not applicable.

Availability of data and materials

The RNA-seq data in this study were available at NCBI Sequence Read Archive with the accession number of SRR9937067–SRR9937074 (https://www.ncbi.nlm.nih.gov/sra/).

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

RW, LW, and HY conceived and designed the study. RW, LW, HM, ML, and ZC performed the experiments. RW, XD, LZ, and YL analyzed the data and drafted the manuscript with LH; RW, LW, HY, and TC wrote the manuscript. All authors read, revised extensively, and gave final approval of the manuscript.

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Figures

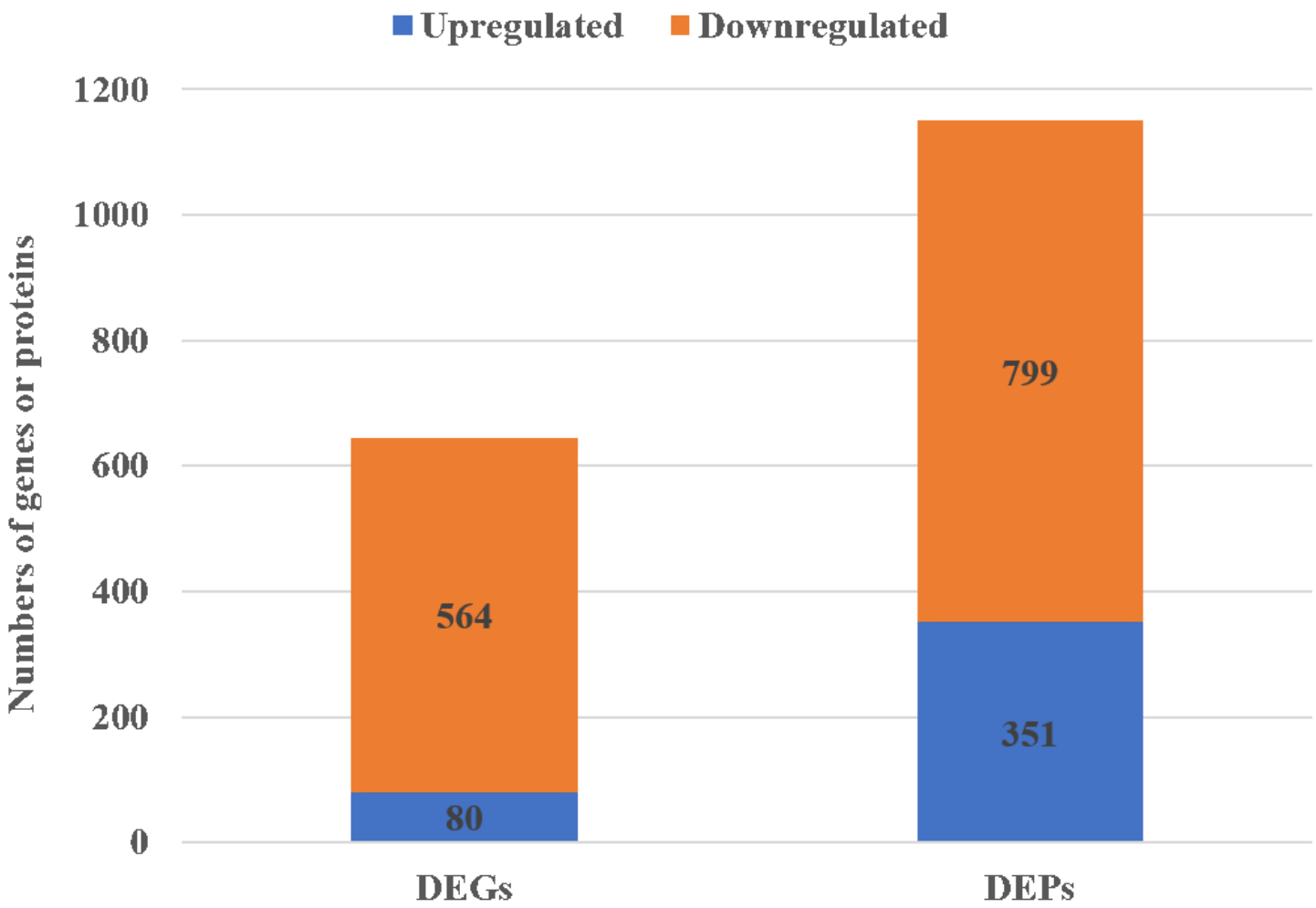

Figure 1

The genes and proteins differentially expressed between the immunized group and the control group.

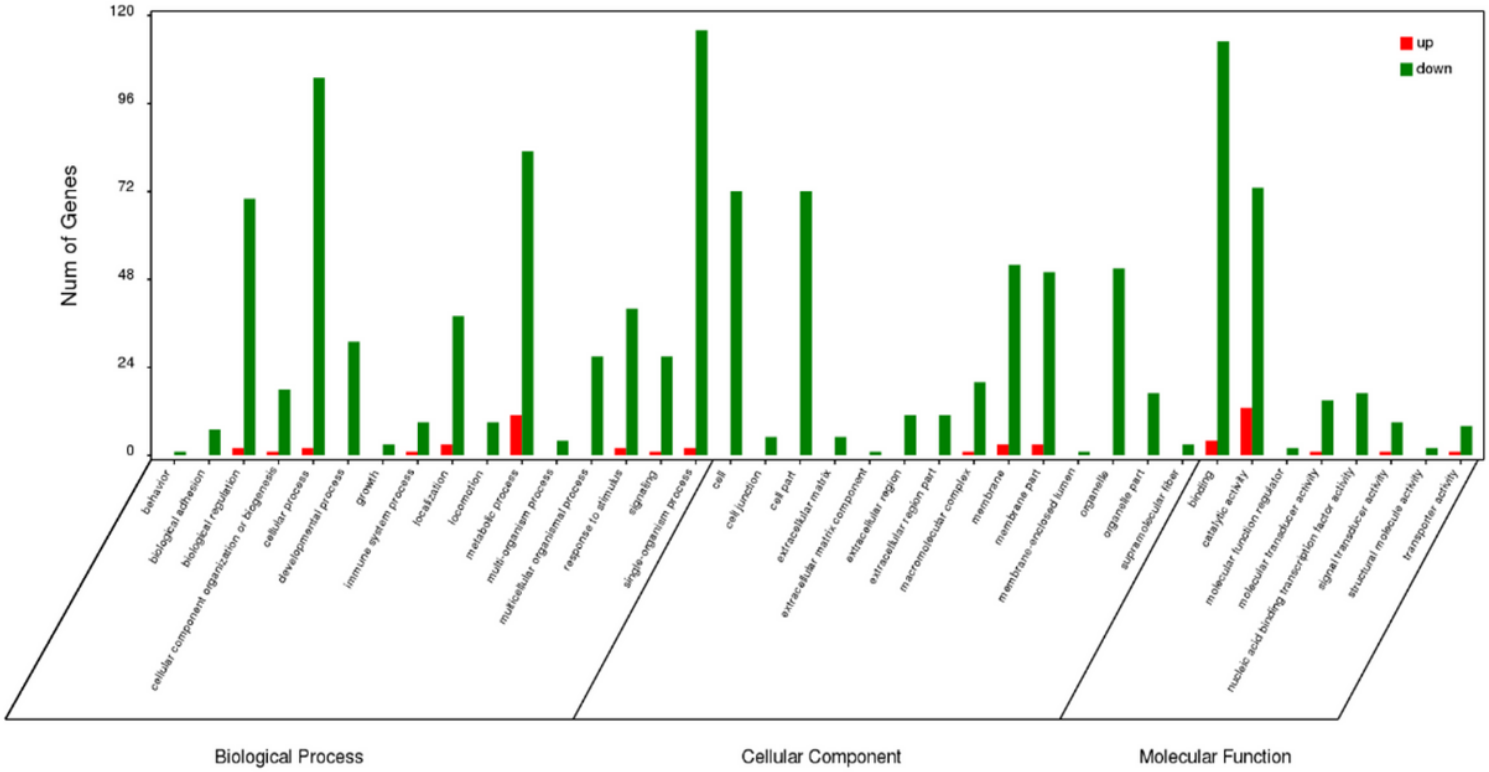

Figure 2

GO classification of genes differentially expressed between the immunized group and the control group.

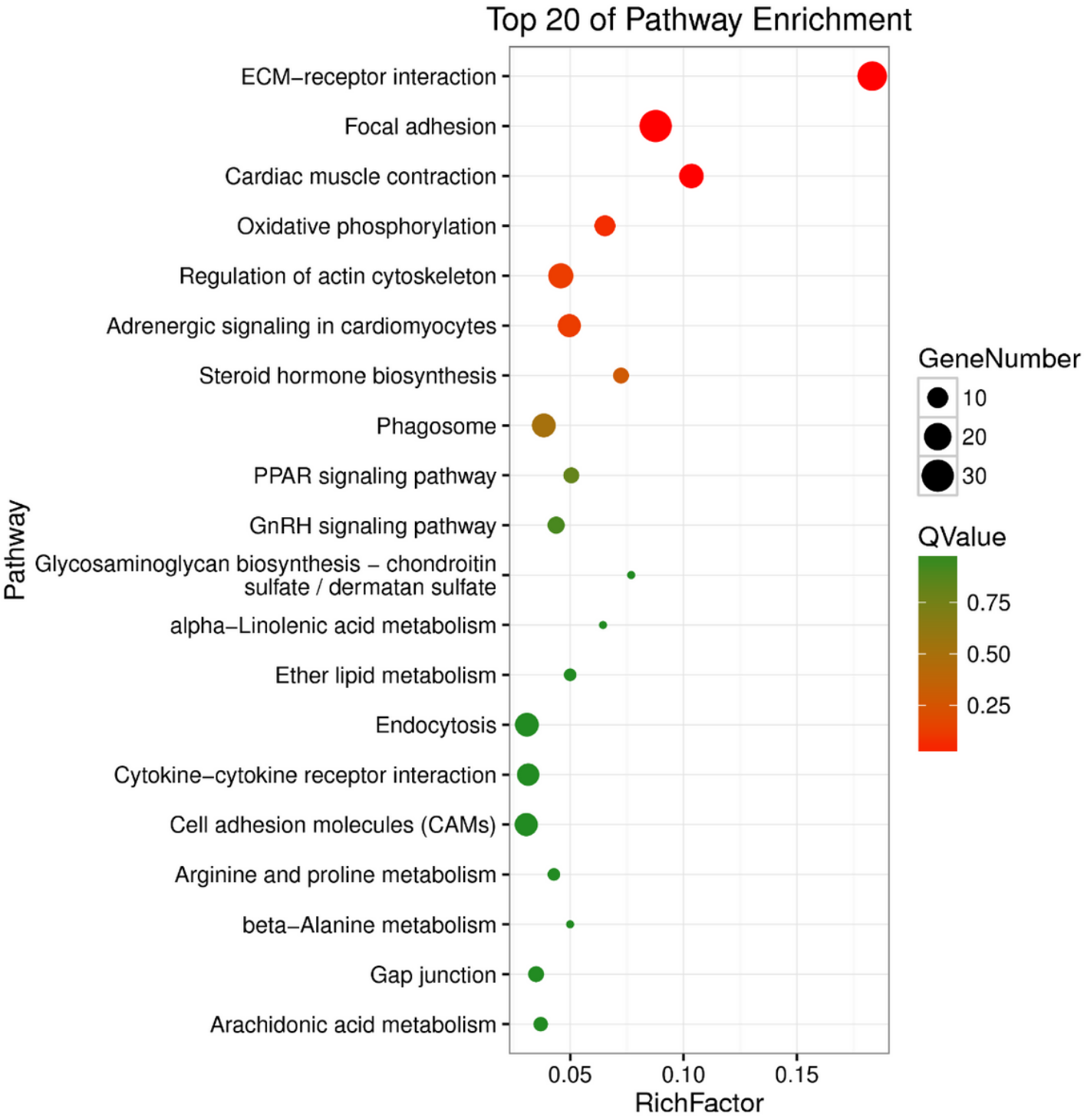

Figure 3

Enrichment of DEGs in KEGG pathways.

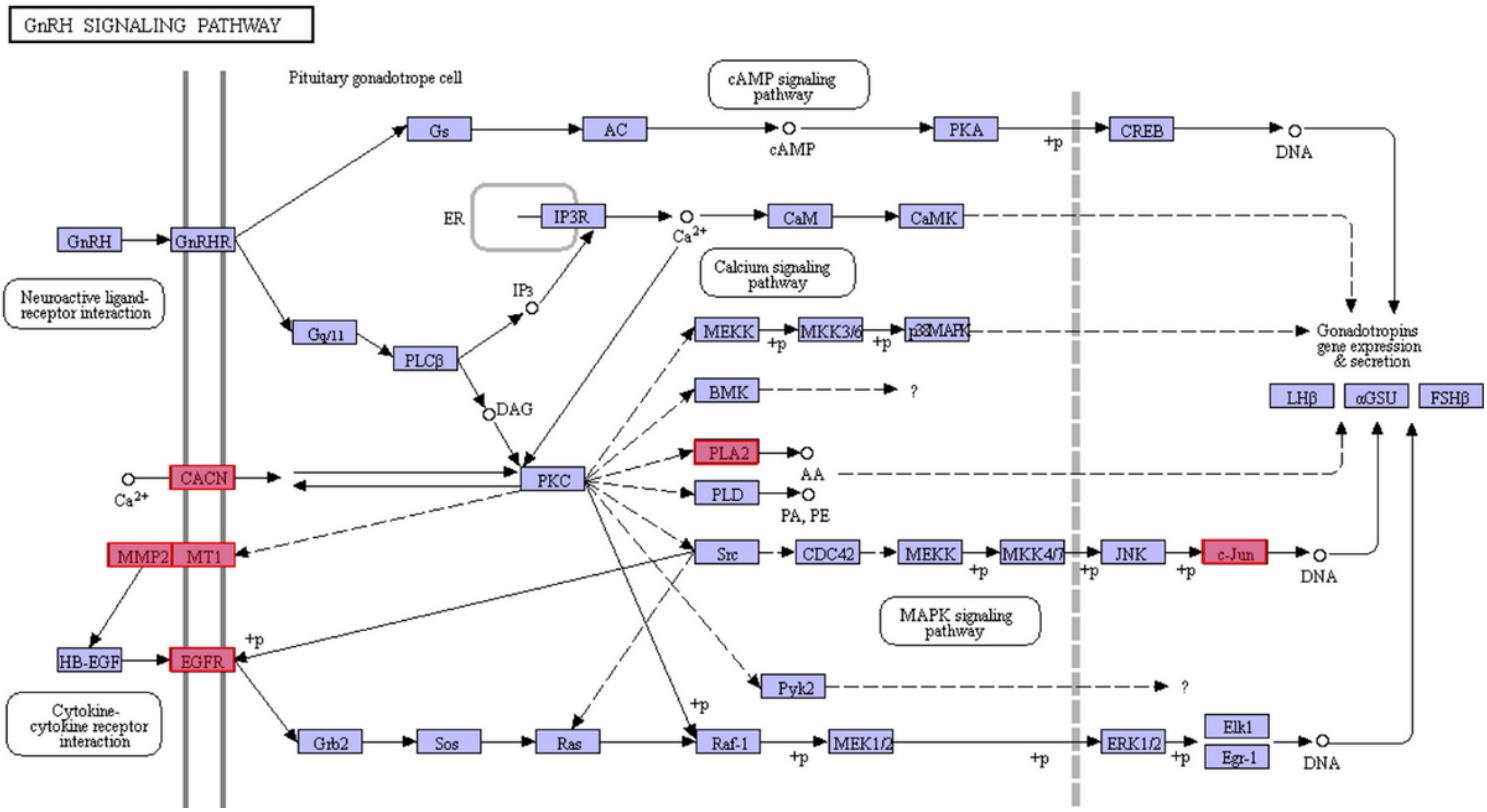

Figure 4

The location of the six genes in the GnRH signaling pathway.

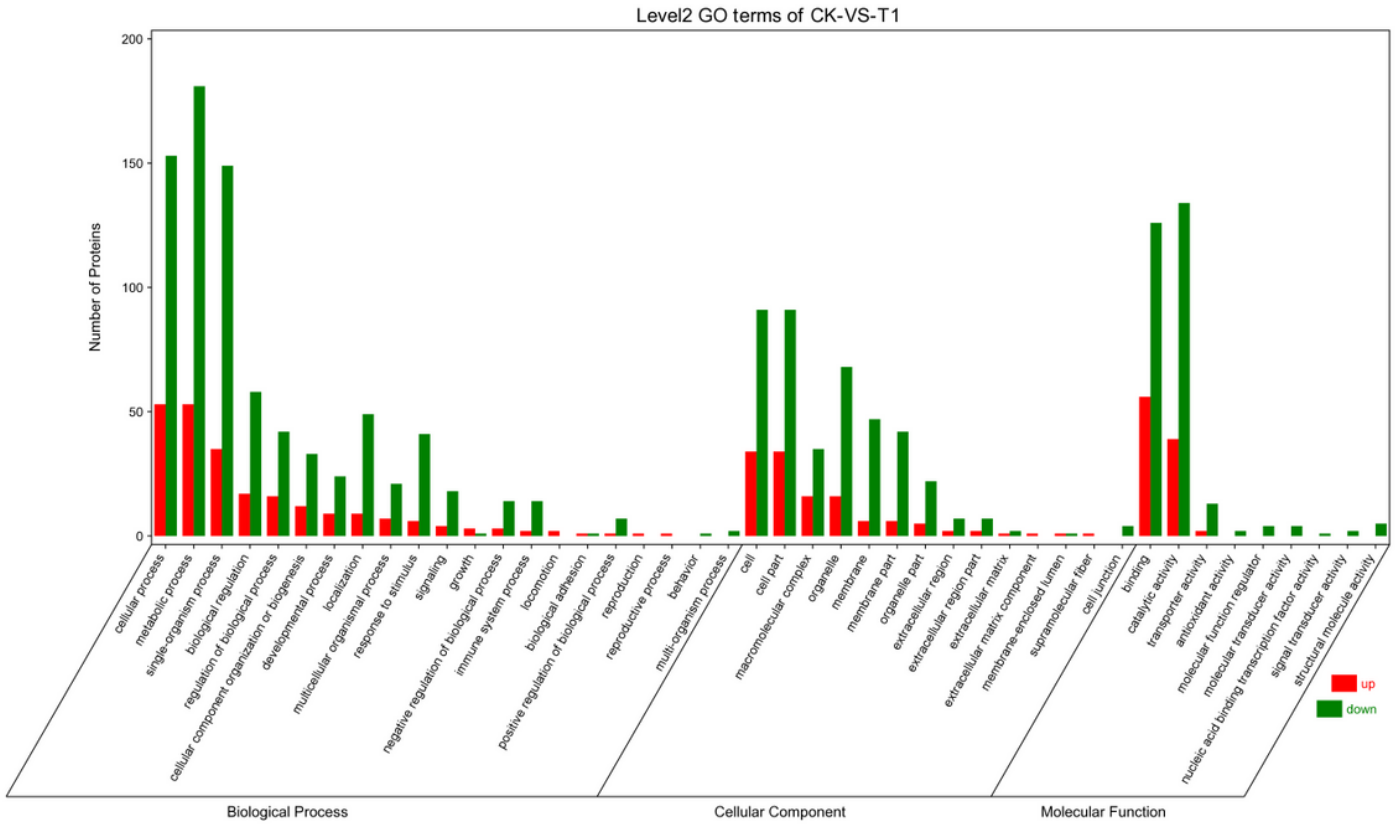

Figure 5

GO classification of DEPs between the immunized group and the control group.

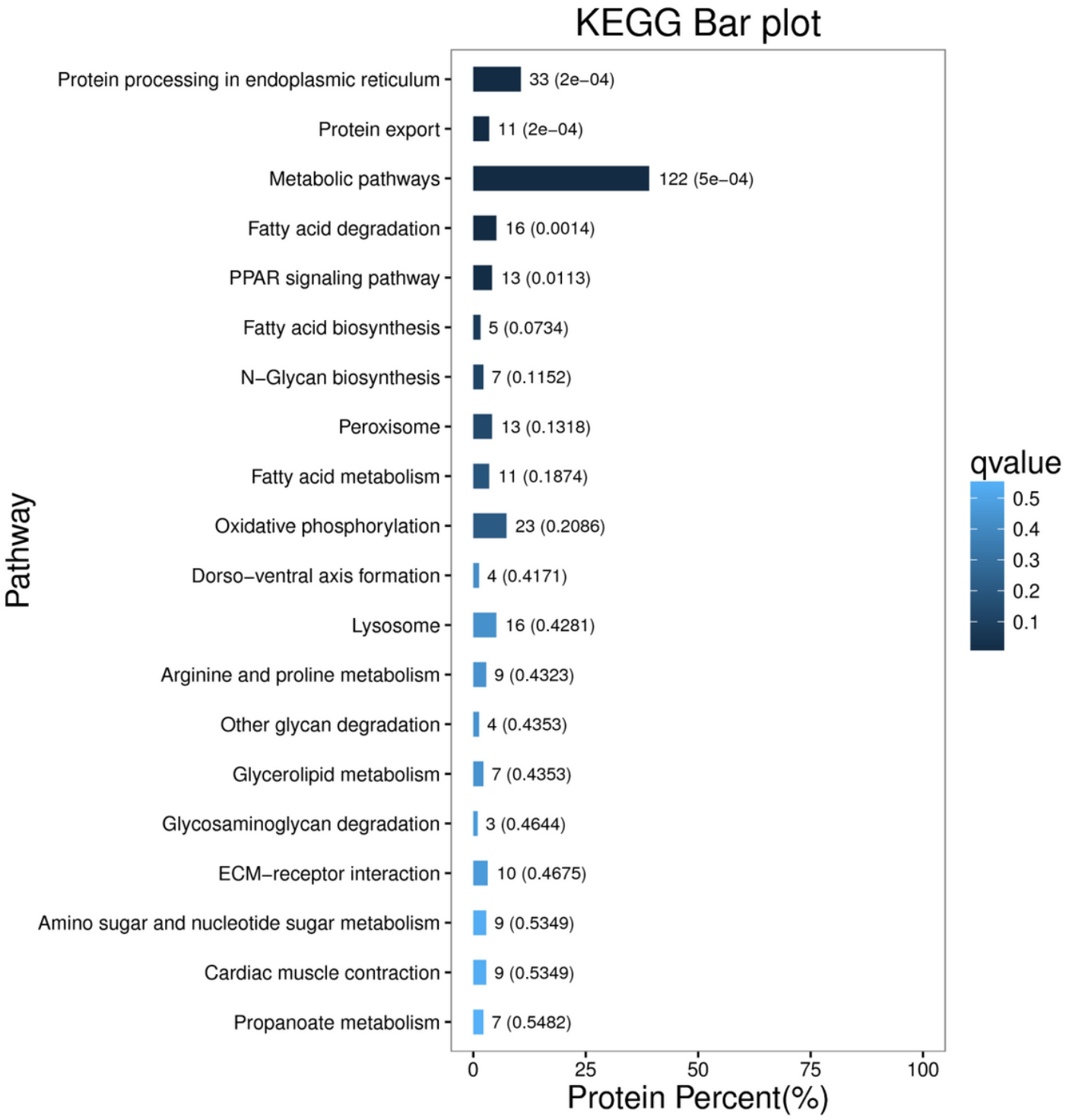

Figure 6

Enrichment of DEPs in KEGG pathways.

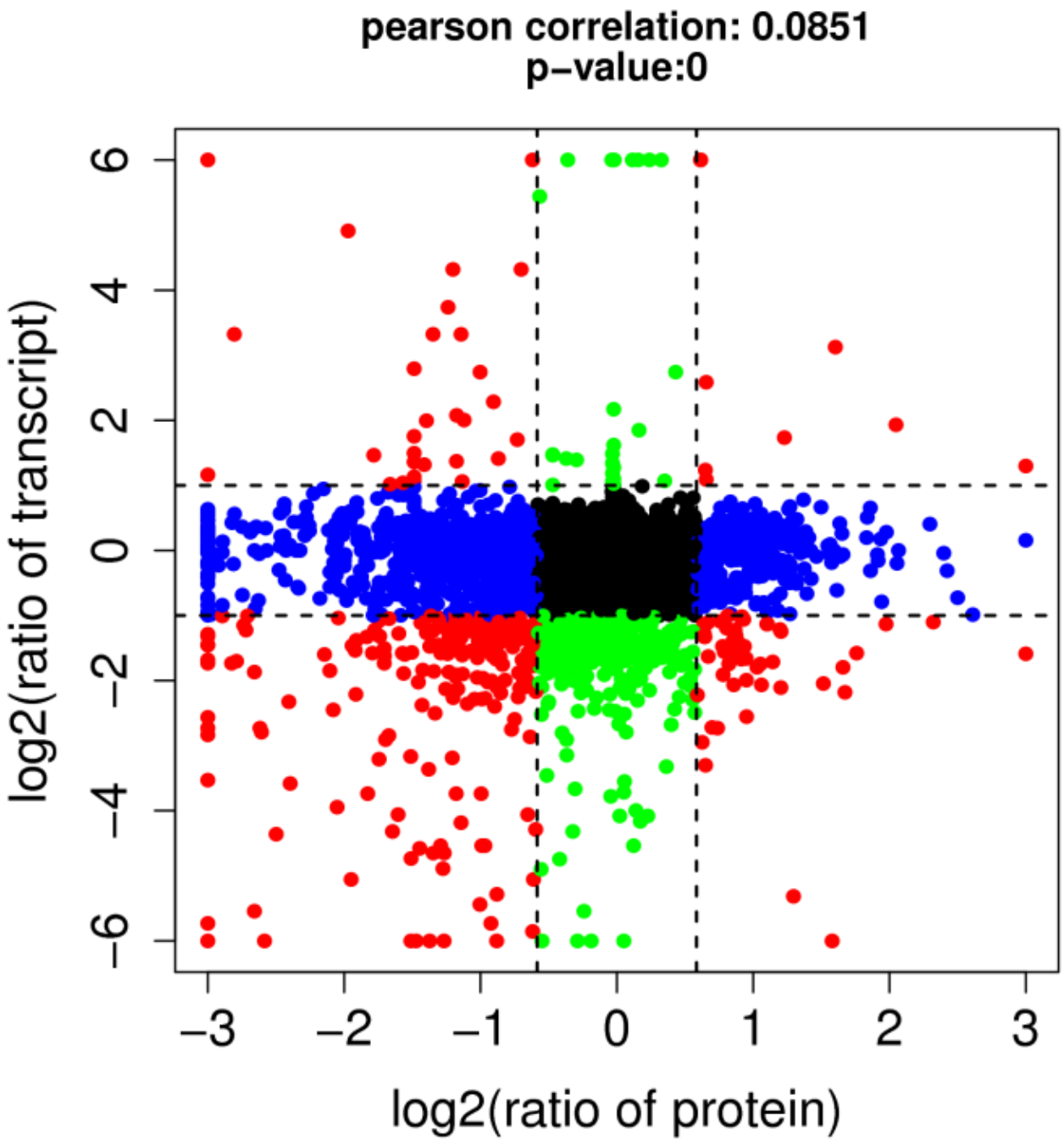

Figure 7

Correlation analysis of the protein and mRNA expression between the immunized group and the control group. The x-axis represents the log2 value of the fold differential gene expression, the y-axis represents the log2 value of the fold differential protein expression, and the dotted line indicates the threshold of significant differential expression.

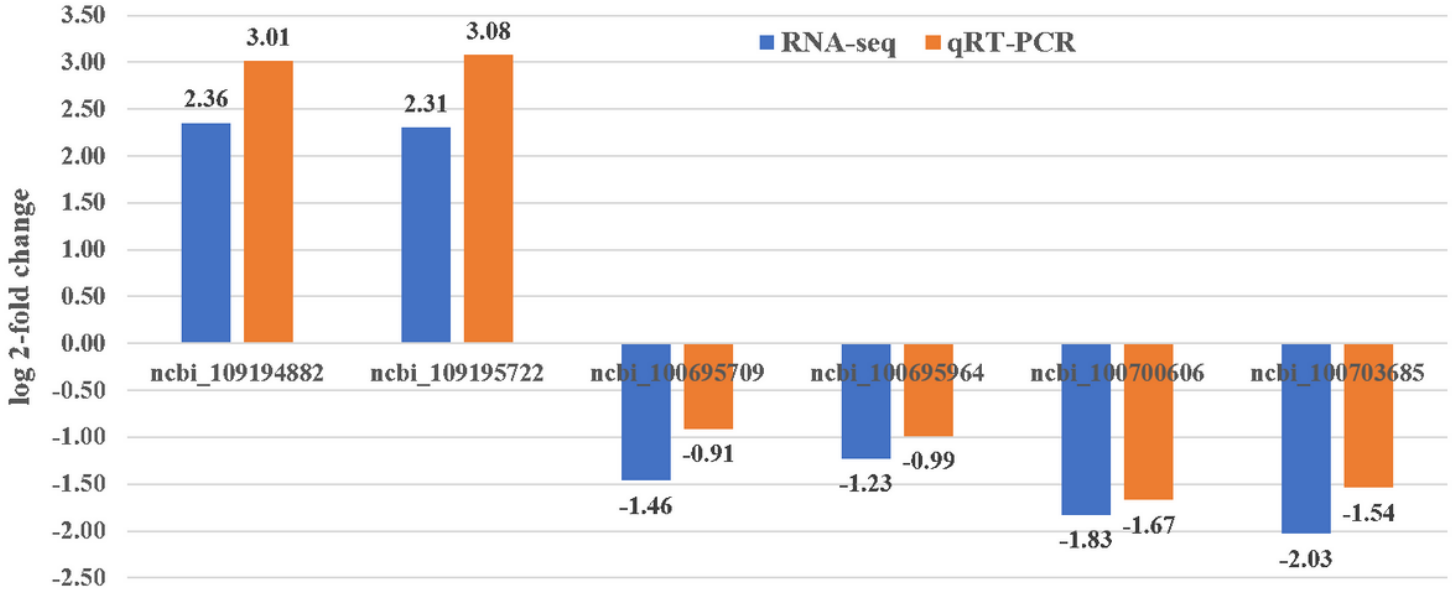

Figure 8

Comparison of the expression profiles of six selected genes as determined by RNA-seq and qRT-PCR.

Supplementary Files

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- NC3RsARRIVEGuidelinesChecklist2014.pdf