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Transcriptome Analysis Reveals Differences in Gene Expression in the Muscle of the Brown-Marbled Grouper (*Epinephelus fuscoguttatus*) with Different Growth Rates

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Abstract: Brown-marbled grouper is one of the most important mariculture species in China and is widely used as a crossbreeding parent in the grouper industry. Enhancing growth rates is a key target in fish breeding, and gaining insight into the underlying mechanisms responsible for growth differences between individuals can aid in the improvement of grouper growth rates. However, the mechanism behind growth differences in this fish remains unclear. We analyzed the transcriptome profiles of muscle tissues between fast- and slow-growing brown-marbled grouper using RNA-seq and identified 77 significantly up-regulated genes and 92 significantly down-regulated genes in the extreme growth groups. Our findings suggest that up-regulated genes such as *ghr* and *tnni2*, as well as down-regulated genes such as *stc2* and *pdp1*, are associated with growth advantages in brown-marbled grouper. We used differentially expressed genes (DEGs) for GO and KEGG enrichment analyses. The results of the GO enrichments showed that the significantly up-regulated genes in the fast-growing group were involved in protein folding, the actin cytoskeleton, the myosin complex, and other processes. The results of the KEGG enrichments showed that the significantly up-regulated genes in the fast-growing group were involved in various pathways such as glycolysis/gluconeogenesis, adipocytokine signaling, MAPK signaling, carbon metabolism, and PI3K-Akt signaling. Additionally, gene set enrichment analysis (GSEA) showed that the PI3K/AKT/mTOR pathway was up-regulated in the fast-growing group, which may be responsible for the higher nutrient absorption efficiency and muscle growth in these fish. Our results contribute to a better understanding of the molecular mechanisms and regulatory pathways underlying fast growth in brown-marbled grouper. However, further studies are necessary to fully elucidate the mechanisms behind growth differences between individuals.

Keywords: brown-marbled grouper; muscle; growth; RNA-seq

Key Contribution: The manuscript presents findings from transcriptome analysis of muscle tissue in fast- and slow-growing brown-marbled grouper, identifying genes and pathways associated with fast growth. The study contributes to a better understanding of the molecular mechanisms and regulatory pathways underlying fast growth in this important mariculture species.

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Citation: Wang, T.; Yang, Y.; Gong, S.; Wu, X.; Zeng, L.; Tao, Y.; Zhong, C.; Song, L.; Liu, X. Transcriptome Analysis Reveals Differences in Gene Expression in the Muscle of the Brown-Marbled Grouper (*Epinephelus fuscoguttatus*) with Different Growth Rates. *Fishes* **2023**, *8*, 309. https://doi.org/10.3390/fishes8060309

Academic Editor: Qiuning Liu

Received: 18 April 2023
Revised: 2 June 2023
Accepted: 6 June 2023
Published: 8 June 2023

1. Introduction

Brown-marbled grouper (*Epinephelus fuscoguttatus*) belongs to the *Epinephelus* genus of the *Epinephelidae* family and is an important, economically valuable, edible fish found in tropical and subtropical seas of Asia [1]. Due to its excellent meat quality, high nutrient

content, and rapid growth, the brown-marbled grouper has a high market value and is widely cultured in southern China [2]. Additionally, its long and stable spawning cycle and high spawning quality and quantity make it an ideal choice as a female parent in crossbreeding programs [3–5]. Currently, the Hulong grouper, the grouper with the largest production in China, is produced by crossing the brown-marbled grouper with the giant grouper (*E. lanceolatus*) [6].

Growth is a critical trait for economically significant aquaculture species, and it mainly depends on the increase in muscle mass. The muscle content can comprise up to 50–70% of the fish's body weight, and it primarily drives the fish's growth [7]. Ray-finned fish's muscle growth differs from that of mammals, with hyperplasia continuing throughout the fish's lifespan. The intricate regulatory mechanisms of muscle growth are mainly regulated by the GH/IGF system, which promotes hyperplasia and hypertrophy through the stimulation of cell proliferation, differentiation, myogenesis, protein synthesis, and protein degradation [8,9]. Environmental factors such as light, temperature, and food supply can also affect muscle growth by influencing the proliferation of myogenic progenitor cells (MPCs), driving hyperplasia [10,11].

It has been reported that in seven species of freshwater fish, muscle growth occurs until approximately 40% of the maximum body length is reached, beyond which hypertrophy becomes the primary driver of growth [12]. Growth signaling pathways such as the GH/IGF axis regulate the synthesis and degradation of protein and control the rate of fiber expansion. The GH/IGF system stimulates the synthesis and secretion of IGF-1 in the liver and other tissues, which then promotes muscle growth through the activation of MAPK/ERK, PI3K/AKT/TOR, and other signaling pathways [13,14]. However, our understanding of the detailed molecular regulatory mechanisms in teleost fish is limited, and the biological functions and roles of many genes involved in fish growth are poorly understood. The analysis of ortholog and paralog genes generated in fish through whole-genome duplication (WGD) further hinders their study [15]. In addition, the genetically based responses of growth to environmental inputs can be highly varied, leading to potentially conflicting results. A more systematic understanding of the genes responsible for muscle growth is needed, and improved knowledge of the molecular genetic basis will provide new opportunities for enhancing muscle growth.

RNA-seq is a recently developed approach that utilizes high-throughput sequencing technologies for transcriptome profiling [16]. Over the past few decades, RNA-seq has been widely employed in aquaculture to identify candidate genes associated with economically important traits. For instance, RNA-seq has been utilized to identify differentially expressed genes involved in disease and stress responses in catfish (*Ictalurus punctatus*) [17,18]. Additionally, RNA-seq has been used to explore differentially expressed genes related to growth in various fish species, such as rainbow trout (*Oncorhynchus mykiss*), largemouth bass (*Micropterus salmoides*), and schizothoracine fish (*Schizothorax prenanti*) [19–21]. Numerous genes and signaling pathways involved in the regulation of the growth process of ray-finned fish have been discovered. In a transcriptome study focusing on muscle growth in mandarin fish (*Siniperca chuatsi*), differentially expressed genes (DEGs) associated with GH-IGF pathways, protein synthesis, ribosome synthesis, and energy metabolism were found to be expressed at significantly higher levels in larger individuals compared to smaller ones [7]. Another study examined muscle and liver tissues of black carps (*Mylopharyngodon piceus*) from the same batch with different growth rates, using RNA-seq to evaluate their growth traits. The findings revealed significant up-regulation of growth-related pathways, including the FoxO signaling pathway, p53 signaling pathway, PI3K-Akt signaling pathway, and insulin signaling pathway, in black carps with fast growth rates [22]. Although the present study serves as a foundation for investigating the growth of the brown-marbled grouper, it is important to note that variations in species or environmental factors may yield different results.

In this study, we employed RNA-seq to analyze the muscle transcriptome of fast- and slow-growing brown-marbled grouper in a half-sibling family, aiming to identify important

candidate genes associated with brown-marbled grouper growth. The results of this study contribute to a better understanding of the mechanisms underlying growth regulation in brown-marbled grouper.

2. Methods

2.1. Ethics Statement

All experiments conducted in the present study were approved by the Animal Care and Use Committee of the School of Life Sciences, Sun Yet-Sen University.

2.2. Experimental Fish and Sample Collection

The brown-marbled groupers were collected from Hainan Chenhai Aquaculture Co., Ltd., a half-sibling family constructed through single-sire and multiple-dam mating. To mitigate the potential impact of gender-related growth differences, we conducted sampling of the brown-marbled groupers after one year of aquaculture cultivation, before the occurrence of sexual reversal. A total of 300 fish were randomly selected and measured for their body length and weight. Subsequently, three fish with the fastest growth rate (average body length: 37.7 ± 0.62 cm; average body weight: 1071.7 ± 5.79 g) and three fish with the slowest growth rate (average body length: 29.7 ± 0.94 cm; average body weight: 393.3 ± 30.35 g) were chosen. The two extreme groups were anesthetized with tricaine eugenol for subsequent sampling, and white muscle was collected from the base of the dorsal fin of the brown-marbled groupers. Precautions were taken to prevent contamination of the muscle tissues with blood vessels and nerve tissues during the sampling process. The tissues were rapidly frozen in liquid nitrogen and stored at −80 °C.

2.3. RNA Extraction and Library Construction

Total RNA from each sample of 6 fish was extracted using the Trizol Reagent (Invitrogen, City of Carlsbad, CA, USA). RNA integrity was assessed using 1% agarose gel electrophoresis after removing the genomic DNA with DNase I (TaKaRa, Dalian, China). RNA quality and quantity were confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, City of Santa Clara, CA, USA) and ND-2000 platform (NanoDrop Technologies, City of Wilmington, DE, USA). mRNA was isolated using oligo (dT) beads and subjected to reverse transcription and fragmentation. Double-stranded cDNA was synthesized using random hexamer primers. Agarose gel electrophoresis was used to select and purify 300–400 bp cDNA fragments, which were then subjected to end repair, "A" base addition, and adapter connection. Library sequencing was performed after amplification with 15 PCR cycles. The cDNA libraries were sequenced on the Illumina HiSeq 2000 platform with a strategy of paired-end 150 bp (PE 150).

2.4. DEG Analysis and GSEA Analysis

Quality filtering was performed using Fastp v0.20.1 [23], and the default settings were used to remove residual adapter sequences from the reads. Illumina-specific adapters were clipped from the reads, and bases with a Phred score less than 20 at the leading and trailing ends were eliminated. Only read pairs with both pairs longer than 36 bp were retained after filtering. The high-quality clean reads were mapped to the brown-marbled grouper genome [24] using HISAT2 v2.1.0 [25]. The uniquely mapped paired-end reads were counted and assigned to genes using FeatureCounts [26] in the Subread package v1.5.0, available on SourceForge. Differential expression analysis was conducted between the fast- and slow-growing brown-marbled grouper samples using the edgeR package (http://www.r-project.org/, accessed on 2 June 2023) [27]. Genes with an absolute log2 fold change (|log2FC|) greater than 1 and a false discovery rate (FDR) less than 0.05 were considered significant differential genes.

The DEGs were compared with the GO database (http://www.geneontology.org/, accessed on 2 June 2023) to obtain a list and count of transcripts associated with GO functions. Hypergeometric tests were conducted to identify significantly enriched GO

entries, as compared to the entire set of transcripts. Similarly, the DEGs were compared with the KEGG database, and hypergeometric tests were used to identify significantly enriched KEGG pathways [28]. The analysis of GO terms and pathways can provide insight into the biological functions of transcripts. All expression data were statistically analyzed and visualized using R (version 4.1.3, https://www.r-project.org, accessed on 2 June 2023).

Gene set enrichment analysis (GSEA) was utilized to determine whether a pre-defined set of genes exhibited statistically significant, concordant differences between the two biological states. The expressed genes were analyzed using GSEA software version 4.1.0 [29] to identify pathways that contributed to differences in growth. The GSEA analysis was conducted according to $|NES| > 1$, NOM p-val < 0.05 and FDR q-val < 0.25 [29].

2.5. Real-Time PCR

To validate the transcriptome data, 25 DEGs were selected as verification genes to confirm the reliability of the RNA-seq results. The expression levels of the selected growth-related genes were verified using real-time PCR (RT-PCR) analysis with three replicates for each RNA sample. Specific primers were designed for each gene using Primer Premier 6.0 (Table S1) [30]. The beta-actin gene was used as an internal control. Each reaction system had a total volume of 10 μL, containing 100 ng of cDNA, 0.5 μL of each primer (10 μM), and 5 μL of Power SYBR Green PCR MasterMix (Vazyme Biotech, Nanjing, China), and the remaining volume was filled with double-distilled water to reach 10 μL (Vazyme Biotech, Nanjing, China). A quantitative fluorescence PCR assay was performed using the LightCyclerR® 480 II Real-Time PCR System (Roche, City of South San Francisco, CA, USA) and 384-well plates with Power SYBR Green PCR MasterMix (Vazyme Biotech, Nanjing, China). Each reaction was repeated three times, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Statistical Analyses

Data analysis was performed using R version 4.1.3, and the results were presented as means ± SEM. The body weights of fish in the fast-growing and slow-growing groups were compared using Student's t-test, with the significance level set at $p < 0.05$.

3. Result

3.1. Next-Generation Sequencing and Mapping to the Reference Genome

In total, transcriptome sequencing of the six samples produced a total of 361,058,385 high-quality clean reads (Table S2). All the clean reads were mapped to the brown-marbled grouper genome using HISAT2 and then compressed and sorted using the SAMtools software. More than 97% of the data from the six samples were mapped to the reference genome.

3.2. Identification of Differentially Expressed Genes

A total of 77 significantly up-regulated genes and 92 significantly down-regulated genes were identified between the fast- and slow-growing groups. In the process of the identification of differential genes, many functional genes related to growth were recognized, such as *myh1*, *myh4*, *tnni2*, *gtr12*, *fmo5*, *ttc9a*, and *ghr*. Based on the results of the analysis, a volcano plot was drawn using R and important genes associated with growth were marked (Figure 1).

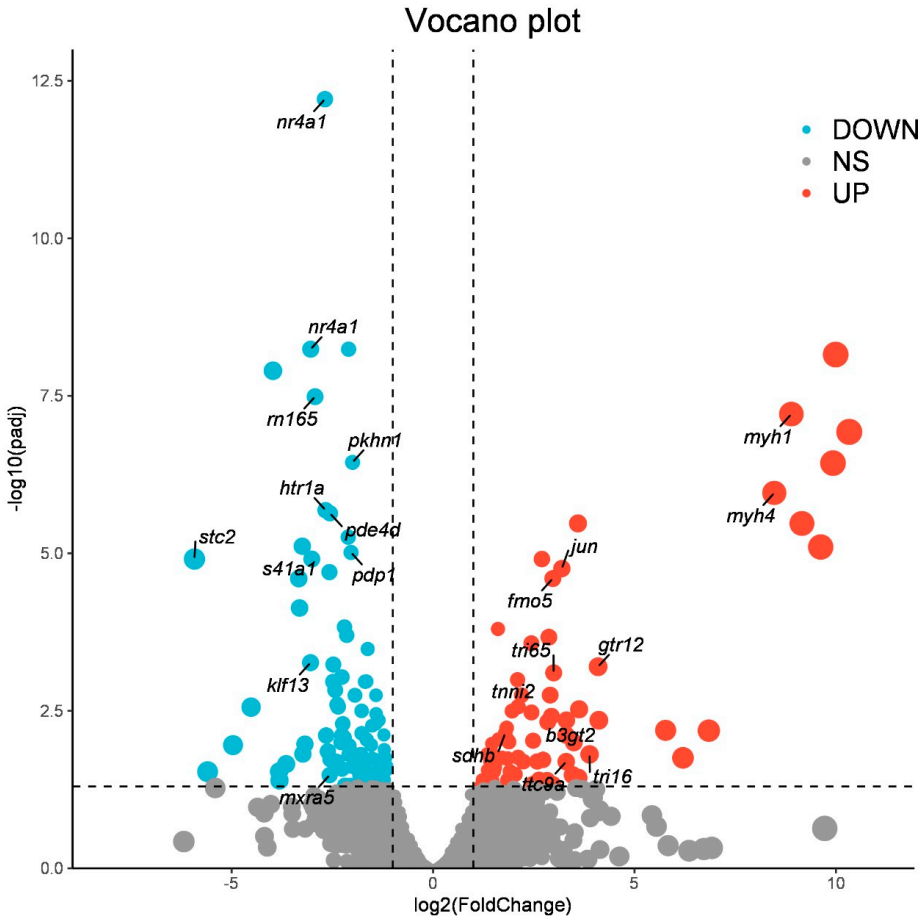

Figure 1. Volcano plot showing the relationship between the magnitude of gene expression change (log2 of fold change) on the x-axis and the statistical significance of this change (log10 of false discovery rate (FDR)) on the y-axis in a comparison between the fast-growing and slow-growing groups. Colored points represent differentially expressed genes (cutoff FDR 0.05) that were either up-regulated (red) or down-regulated (blue) in the fast-growing group compared with the slow-growing group. In the volcano plot, important genes associated with growth are marked, and the size of the points represents the value of gene expression change (log2 of fold change).

3.3. Enrichment Analysis of GO and KEGG Pathways on the Basis of DEGs

GO annotation was used to categorize the 110 DEGs into biological process, cell composition, and molecular function groups, which were further classified into 27 functional categories across 3 ontologies. The cellular component, molecular function, and biological process groups contained nine functional categories each (Figure S1). Notably, significant changes were observed in the myosin complex, cytoskeleton, actin cytoskeleton (cellular component), and motor activity (molecular function) based on the results of the GO analysis (Figure S2).

After excluding pathways associated with human disease, KEGG pathway analysis identified 125 DEGs enriched in various metabolic and organismal system pathways. The major pathways related to growth included steroid biosynthesis (ko00100), glycolysis/gluconeogenesis (ko00010), carbon metabolism (ko01200), the biosynthesis of amino acids (ko01230), MAPK signaling pathway (ko04010), the PI3K-Akt signaling pathway (ko04151), and the glucagon signaling pathway (ko04922) (Figure 2 and Table 1). These significant pathway enrichments provide insight into the molecular mechanisms underlying growth-related traits in the brown-marbled grouper.

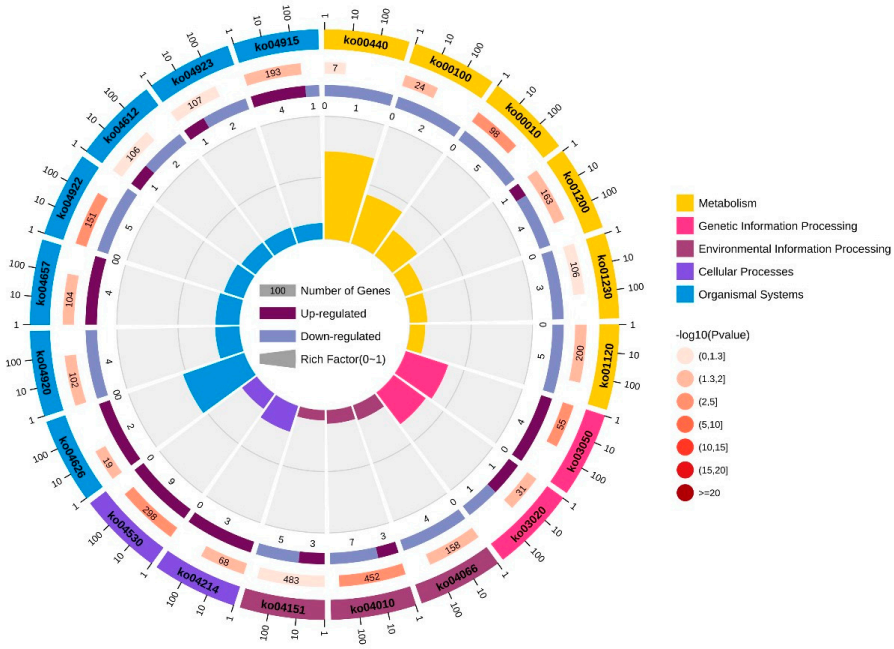

Figure 2. The top 20 pathways enriched among the 106 differentially expressed genes identified via KEGG after eliminating pathways associated with human disease. Circle 1: The top 20 pathways in the enrichment analysis, where the outside circle is the coordinate scale for the number of DEGs. Different colors represent different classes. Circle 2: The number of genes in the background category and their corresponding *p*-values are shown. Longer bars represent a higher number of genes, while a smaller *p*-value is indicated by a more intense red color. Circle 3: The number of DEGs in the pathway. Circle 4: The Rich_Factor value of each pathway.

Table 1. The top 20 pathways enriched among the 106 differentially expressed genes identified via KEGG after eliminating pathways associated with human disease t.

Pathway ID	KEGG_B_Class	Pathway
ko00440	Metabolism of other amino acids	Phosphonate and phosphinate metabolism
ko00100	Lipid metabolism	Steroid biosynthesis
ko00010	Carbohydrate metabolism	Glycolysis/gluconeogenesis
ko01200	Global and overview maps	Carbon metabolism
ko01230	Global and overview maps	Biosynthesis of amino acids
ko01120	Global and overview maps	Microbial metabolism in diverse environments
ko03050	Folding, sorting, and degradation	Proteasome
ko03020	Transcription	RNA polymerase
ko04066	Signal transduction	HIF-1 signaling pathway
ko04010	Signal transduction	MAPK signaling pathway
ko04151	Signal transduction	PI3K-Akt signaling pathway
ko04214	Cell growth and death	Apoptosis—fly
ko04530	Cellular community—eukaryotes	Tight junction
ko04626	Environmental adaptation	Plant–pathogen interaction
ko04920	Endocrine system	Adipocytokine signaling pathway
ko04657	Immune system	IL-17 signaling pathway
ko04922	Endocrine system	Glucagon signaling pathway
ko04612	Immune system	Antigen processing and presentation
ko04923	Endocrine system	Regulation of lipolysis in adipocyte
ko04915	Endocrine system	Estrogen signaling pathway

Note: These pathways are ranked in ascending order, with the top 20 pathways having the lowest *p*-values.

3.4. GSEA Analysis

A total of 14 gene sets were found that were significantly enriched for differentially expressed genes in the fast-growing group, as compared with the slow-growing group. The full list of significantly enriched gene sets can be found in Table 2. Of these, seven gene sets were significantly up-regulated and seven gene sets were down-regulated in the fast-growing group. The important pathways which were related to growth and enriched in the fast- and slow-growing groups are shown in Figure 3.

Table 2. Top down-regulated gene sets from the GSEA analysis.

GS	Biological Features	Number of Genes in Set	Size	ES (Fast vs. Slow)	NES (Fast vs. Slow)	Nominal *p*-Value	FDR *q*-Value
UPSET							
MYOGENESIS	Genes involved in the development of skeletal muscle (myogenesis).	200	114	0.514	2.189	<0.001	<0.001
EPITHELIAL_MESENCHYMAL_TRANSITION	Genes defining epithelial–mesenchymal transition, as in wound healing, fibrosis, and metastasis.	200	89	0.430	1.782	<0.001	0.013
ESTROGEN_RESPONSE_EARLY	Genes defining early response to estrogen.	200	78	0.429	1.731	0.003	0.019
APICAL_JUNCTION	Genes encoding components of the apical junction complex.	200	91	0.418	1.707	0.001	0.021
PI3K_AKT_MTOR_SIGNALING	Genes up-regulated by activation of the PI3K/AKT/mTOR pathway.	105	43	0.435	1.558	0.018	0.068
MITOTIC_SPINDLE	Genes important for mitotic spindle assembly.	199	99	0.369	1.534	0.007	0.069
HYPOXIA	Genes up-regulated in response to low oxygen levels (hypoxia).	200	95	0.348	1.436	0.020	0.134
DOWNSET							
MYC_TARGETS_V1	A subgroup of genes regulated by MYC-version 1 (v1).	200	83	−0.507	−2.327	0.000	0.000
MYC_TARGETS_V2	A subgroup of genes regulated by MYC-version 2 (v2).	58	28	−0.627	−2.210	0.000	0.000
UNFOLDED_PROTEIN_RESPONSE	Genes up-regulated during unfolded protein response, a cellular stress response related to the endoplasmic reticulum.	113	59	−0.416	−1.772	0.000	0.010
OXIDATIVE_PHOSPHORYLATION	Genes encoding proteins involved in oxidative phosphorylation.	200	109	−0.343	−1.634	0.004	0.027
MTORC1_SIGNALING	Genes up-regulated through activation of the mTORC1 complex.	200	98	−0.323	−1.504	0.004	0.066
DNA_REPAIR	Genes involved in DNA repair.	150	64	−0.341	−1.482	0.018	0.066
E2F_TARGETS	Genes encoding cell-cycle-related targets of E2F transcription factors.	200	69	−0.320	−1.414	0.026	0.095

GS: gene set name; SIZE: number of genes in the gene set after filtering out those genes not included in the expression dataset; ES: enrichment score for the gene set; NES: normalized enrichment score; NOM *p*-value (<0.05): the statistical significance of the enrichment score; FDR q-value (<0.25): false discovery rate.

Compared with the slow-growing group, the gene set involved in the development of skeletal muscle (myogenesis), activation of the PI3K/AKT/mTOR pathway (PI3K-Akt-mTOR signaling), and mitotic spindle assembly (mitotic spindle) was significantly up-regulated in the fast-growing group. Compared with the slow-growing group, the gene set encoding proteins involved in oxidative phosphorylation (oxidative phosphorylation), activation of the mTORC1 complex (mTORC1 signaling), and encoding of the cell-cycle-related targets of E2F transcription factors (E2F targets) was significantly up-regulated in the fast-growing group. The heat map of the top 50 features for each phenotype in the GSEA_data is shown in Figure S3.

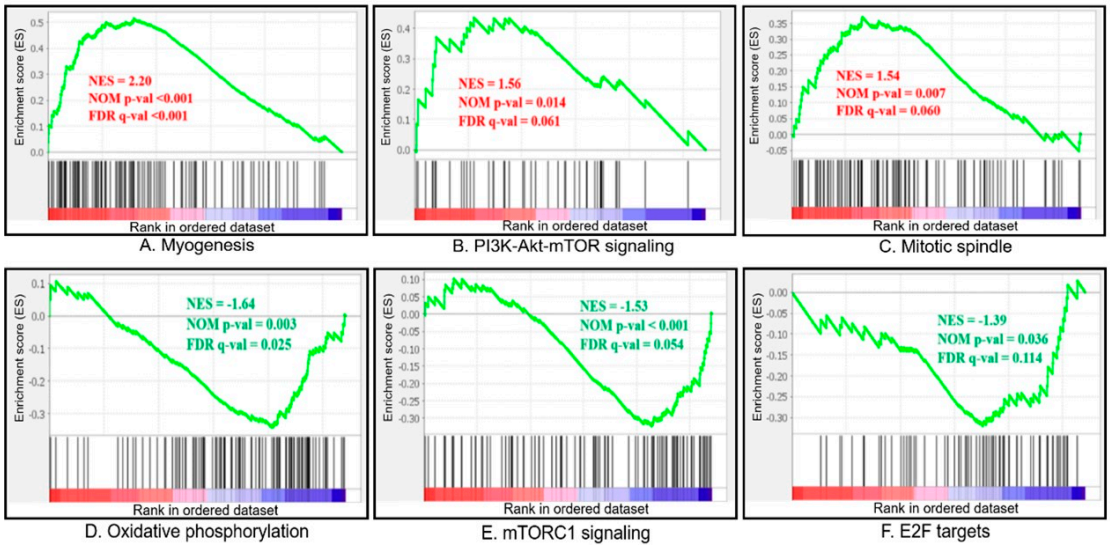

Figure 3. Gene set enrichment analysis (GSEA) enrichment plots of representative gene sets from Table 2: myogenesis (**A**), PI3K-Akt-mTOR signaling (**B**), mitotic spindle (**C**), oxidative phosphorylation (**D**), mTORC1 signaling (**E**), E2F targets (**F**). NES, normalized enrichment score; NOM *p*-val, the statistical significance of the enrichment score; FDR q-val, false discovery rate. Positive and negative NES indicate higher and lower expression in the fast-growing group, respectively.

3.5. qPCR

To verify the accuracy of the RNA-seq data, we screened 25 DEGs involved in muscle contraction (*myh1*, *myh4*), growth hormone receptor (*ghr*), Troponin I, fast skeletal muscle (*tnni2*), facilitated glucose transporter member 12 (*gtr12*), skeletal development (*ttc9a*), glycolysis (*pdp1*), the citric acid cycle (*sdhb*), beta-1,3-galactosyltransferase 2 (*b3gt2*), immunity (*mxra5*), and other processes (Figure S4). The gene expression changes obtained via qPCR were remarkably consistent with those obtained from the RNA-seq results, with R^2 values of 0.9043 (Figure 4). Therefore, the qPCR results confirmed the reliability and accuracy of the RNA-seq data.

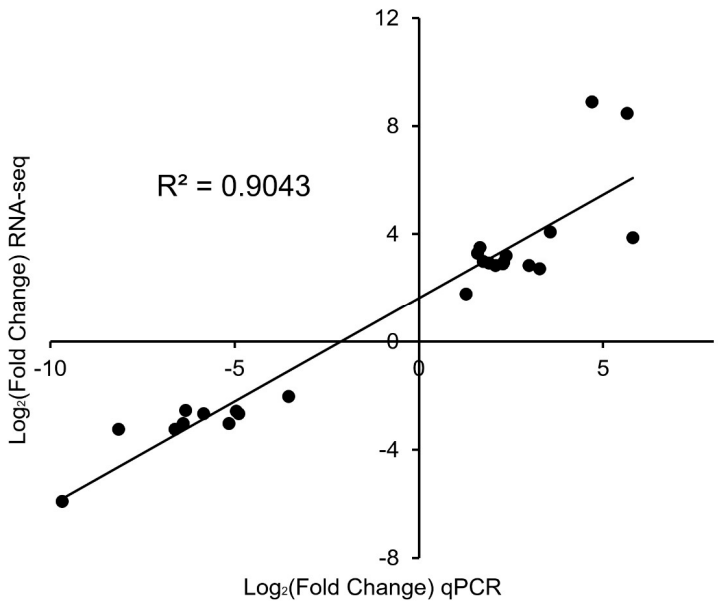

Figure 4. Comparison of gene expression changes measured via RNA-seq and qPCR. The $\log_2$ (fold change) values from the qPCR results are plotted on the x-axis, while those from the RNA-seq results are plotted on the y-axis. The correlation between the two methods is shown.

4. Discussion

Mammalian muscle growth is influenced by various factors, including metabolic, behavioral, and reproductive activities [31]. Similarly, ray-finned fish muscle growth is a complex process, which is controlled by genes, environment, diet, and other factors [32]. We conducted this study after finding that fast- and slow-growing brown-marbled grouper developed differently during cultivation. This study is the first to report on the difference in the brown-marbled grouper muscle transcriptome between fast- and slow-growing groups. Using the resulting muscle transcriptome sequences, we obtained the DEGs of the fast- and slow-growing brown-marbled grouper which potentially influence muscle growth. GO, KEGG, and GSEA analyses were used to explore closely related signaling pathways that may contribute to growth differences.

In our experiments, we found that the genes *ghr* and *tnni2* were significantly up-regulated in the fast-growing group compared with the slow-growing group. *ghr* (the growth hormone receptor) is a cell membrane receptor that specifically binds to growth hormone (GH). Upon binding, *ghr* initiates a phosphorylation cascade that regulates signaling and gene expression, ultimately regulating various physiological functions such as growth, metabolism, appetite, reproduction, and social behavior [33,34]. Based on a study of Rohu (*Labeo rohita*), it was found that several molecules have agonistic effects on the growth hormone receptor protein, and these molecules can be utilized to enhance ray-finned fish growth [35]. *tnni2* is a member of the troponin I gene family that encodes a fast-twitch skeletal muscle protein. It is a constituent of the troponin complex, which comprises troponin T, troponin C, and troponin I subunits [36]. This complex, along with tropomyosin, plays a crucial role in regulating striated muscle contraction in a calcium-dependent manner. Studies on mice showed that *tnni2* knock-out affects bone development in mice, leading to exhibit growth retardation more frequently than wild-type mice [37]. *stc2* (mammalian stanniocalcin-2) is a secreted polypeptide which prevents the PAPP-A cleavage of insulin-like growth factor-binding protein (IGFBP)-4 and, hence, its release within tissues of bioactive IGF, required for normal growth [38]. *stc2* showed down-regulated expression in the fast-growing group, which indicated that low levels of expression were more beneficial for grouper growth. Furthermore, among the differentially expressed genes, we also identified several genes that may be associated with the growth of brown-marbled grouper, such as *pdp1*, *g6pi*, and *s27a2*. *pdp1*, a transcription factor found in the muscle activator region, has been shown to play a role in regulating the expression of the tropomyosin I gene in somatic wall and pharyngeal muscles. This is achieved through binding to specific DNA sequences within the muscle activator that are essential for activation function [39]. *g6pi* is involved in glycolysis [40], and *s27a2* is involved in fatty acid synthesis [41]. Further research is needed to validate the associations between these genes and muscle growth in brown-marbled grouper.

Both the GO and KEGG enrichment analyses revealed that the DEGs were enriched in muscle growth synthesis and in protein and carbohydrate metabolism pathways. In general, muscle growth is associated with increased protein anabolism and glycogen anabolism [42]. Although the GH/IGF-axis-related pathway was not observed in the differential gene enrichment results, its downstream-related phosphonate and phosphinate metabolism, glucose metabolism, carbon metabolism, and amino acid biosynthesis pathways were observed in the KEGG pathway enrichment analysis. In the Hulong grouper (*E. fuscogutatus* ♀× *E. lanceolatus* ♂), the growth advantage appears to be associated with the up-regulation of DEGs related to the GH/IGF axis and its downstream signaling pathways, which enhance protein and glycogen synthesis [4].

In this study, the KEGG pathway enrichment analysis revealed that certain DEGs were enriched in the PI3K-Akt signaling pathway. Furthermore, the GSEA analysis indicated a significant up-regulation of the PI3K/AKT/mTOR pathway in the fast-growing group compared to the slow-growing group. Prior studies have established that the core components of the PI3K/AKT/mTOR signaling pathway include phosphatidylinositol 3-kinase (PI3Ks) and its downstream targets, Protein Kinase B (AKT) and the mammalian target of

rapamycin (mTOR) [31]. mTOR is a downstream effector protein of the PI3K protein kinase family signaling pathway. In mammals, it is present in two separate complexes, mTORC1 and mTORC2, which are differentiated by their respective accessory proteins, raptor and rictor [43].

mTORC1 is a key regulator of nutrient storage that promotes the synthesis of enzymes involved in glucose biosynthesis, as well as the production of proteins, lipids, nucleotides, and ATP, thereby facilitating muscle growth [44,45]. Additionally, the PI3K/AKT/mTOR cascade serves as a crucial effector of insulin signaling and plays a major role in inducing glycolysis and lipogenesis while simultaneously repressing glycogenolysis [46]. Activation of the PI3K/AKT/mTOR pathway through feeding can stimulate mRNA translation and protein synthesis while also regulating a variety of processes, such as nutrient absorption, cell growth, proliferation, and anabolism [44,47]. Insulin binds to cell surface receptors to activate the PI3K-AKT pathway via IRS1. AKT promotes glucose absorption and activates mTORC1 activity through the AKT-TSC1/2-RheB-mTORC1 pathway [48]. In a recent study involving crucian carp, the heterozygous deletion of pik3r1 was utilized to enhance PI3K/AKT/mTOR signaling pathway activity, leading to improvements in somatic cell growth and food utilization efficiency [49]. Some studies have also shown that the swimming training of zebrafish (*Danio rerio*) can increase the activation and expression of the mTOR pathway in the skeletal muscle and then increase the growth of zebrafish skeletal muscle [50]. Other studies have shown that amino acid supplementation in mice can stimulate the mTOR pathway to optimize muscle synthesis after feeding but does not increase the speed of protein synthesis [51]. Juvenile rainbow trout can regulate the activation of the mTOR pathway through feeding, subsequently enhancing protein synthesis [52,53]. Therefore, compared with the slow-growing group, the PI3K/AKT/mTOR pathway of the fast-growing group was significantly up-regulated, possibly indicating that the expression of this signaling pathway led to a higher nutrient absorption efficiency in the fast-growing group. In the growth process of grouper, nutrition, digestion, and absorption play important roles. Higher nutrition utilization is beneficial for the growth of muscle and the increase in the body length and weight of ray-finned fish.

5. Conclusions

In the present experiments, RNA samples from brown-marbled grouper muscle were subjected to RNA-seq analysis, and many DEGs were obtained. Genes such as *myh1*, *myh4*, *ghr*, *gtr12*, and *tnni2* were up-regulated in the fast-growing fish, while *sct2*, *pdp1*, *mxra5*, and others were down-regulated, which might promote muscle growth. GO and KEGG enrichment analyses revealed significant differential gene involvement in steroid biosynthesis, carbohydrate metabolism, glycolysis, gluconeogenesis, skeletal system development, motor activity, and cellular macromolecule biosynthetic processes. The GSEA analysis results showed that the PI3K/AKT/mTOR pathway was significantly up-regulated in the fast-growing group, indicating that fast-growing groupers have higher nutritional utilization. The transcriptome sequences generated in this study have great potential to facilitate gene function analysis and molecular marker development based on gene sequence data, accelerating research progress. Furthermore, the availability of sample data can enable more in-depth investigation of the growth and other traits of brown-marbled grouper.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8060309/s1, Table S1: The specific primers for RT-PCR; Table S2: Summary of transcriptome data generated from the brown-marbled grouper samples; Figure S1: GO assignment of the differentially expressed genes; Figure S2: Bar plots of down-regulated genes in fast-growing grouper of GO functional enrichment analyses; Figure S3: Bar plots of up-regulated genes in fast-growing grouper of GO functional enrichment analyses; Figure S4: A: Bar plot of up-regulated genes in the fast-growing group based on KEGG enrichment analyses. B: Bar plot of down-regulated genes in the fast-growing group based on KEGG enrichment analyses; Figure S5: Heat map of the top 50 features for each phenotype in the GSEA data; Figure S6: L represent the slow-growing group; H represent the fast-growing group.

Author Contributions: T.W. and Y.Y. conceived and designed the study. L.Z. and Y.T. were responsible for the fish rearing and sampling. C.Z. and L.S. performed the RNA-seq and qPCR analyses, respectively, inferring the biological significance of the DE genes. T.W., S.G. and X.W. analyzed the data and wrote the manuscript. X.L. made modifications to the article and provided guidance for the experiment. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (U22A20531, 32273132), China Agriculture Research System of MOF and MARA (CARS-47), National Key Research and Development Program (2018YFD0900203), and Project of Hainan Academician Team Innovation Center (YSPTZX202122).

Institutional Review Board Statement: The Animal Care and Use Committee in School of Life Sciences, Sun Yet-Sen University SYSU-IACUC-2022-B0131.

Data Availability Statement: The raw sequence reads were deposited in NCBI's Short Read Archive (SRR19851482-SRR19851487) under the BioProject accession number PRJNA850911.

Conflicts of Interest: The authors declare that they have no competing interest.

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