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Short communication

Greater potency of adipocytes compared with preadipocytes under lipopolysaccharide exposure in grass carp *Ctenopharyngodon idella*

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ABSTRACT

Excessive body fat is a chronic inflammatory disorder. In this process, white adipose tissue (WAT) performs immune activities because of the dysregulated expression of adipokines. Excessive fat is accumulated in farmed fish, thereby threatening fish health. Studies have shown that adipose tissue is also an active immune organ in fish, capable of participating in and influencing immune responses. Adipocytes are the main cellular component of adipose tissue; however, little is known about the relationship between adipocyte and inflammation in fish. In this study, we analyzed transcriptome changes during adipogenesis in the primary culture of grass carp adipocytes using bioinformatics. The results showed that inflammatory signaling pathway may be activated during grass carp adipocyte differentiation, such as NFκB signaling pathway, Toll-like receptor signaling pathway and Adipocytokine signaling pathway, indicating that grass carp adipocytes have immune activities. Exposure to LPS induced expression of adipokines genes in adipocytes and preadipocytes, including NF-kB, IL-6, MCP-1 and TNFα, suggesting that preadipocytes and adipocytes both have immune response and the immune activity is conserved in vertebrates white adipocytes. Further study found that these immune marker genes were higher expressed in adipocytes compared with preadipocytes in LPS-induced inflammation. In summary, adipocyte should be considered as an active immune site in fish. Adipocytes have greater potency compared with preadipocytes in LPS-induced inflammation. This study indicated that adipocytes and preadipocytes may have different contribution in inflammation.

1. Introduction

White adipose tissue (WAT) is not only an energy storage organ but also functions as an endocrine and secretory organ that secretes various multifunctional molecules termed adipokines [1,2]. Excess adiposity or adipocyte dysfunction can cause the dysregulated expression of these factors, contributing to the pathogenesis of various disease processes through altered immune responses [3]. Hence, adipose tissue is increasingly seen as playing an important role in immune function [4–6].

WAT contains many cell types, including preadipocytes, adipocytes, endothelial cells, fibroblasts, and immune cells such as macrophages and T cells. They are involved in autocrine, paracrine, and endocrine processes and cross-talks [7]. Adipocytes, the main cellular component of adipose tissue, are considered as the major adipogenic cell type secreting proinflammatory cytokines in WAT, and the adipocyte hypertrophy is thought to be associated with deleterious effects on inflammation [8]. Moreover, many studies have shown that preadipocytes can also be activated and acquire proinflammatory features [9,10], being a major source of proinflammatory cytokines or chemokines that recruit macrophages in adipose tissue [11]. However, increasing evidences indicate that preadipocytes and adipocytes have different inflammatory response. Under hypoxia conditions, preadipocytes from WAT with a higher inflammatory response than adipocytes, leading the expression and secretion of inflammatory markers, such as IL-6, MCP-1 and TNF-α [12,13]. Preadipocytes produce higher levels of proinflammatory cytokines compared with adipocytes, leading to endothelial activation in coculture as demonstrated in TNF-α exposure [10]. These reports suggest that preadipocytes and adipocytes have different contribution to the pool of adipokines, resulting in different inflammatory response.

Farmed fish in aquaculture is based on feeds with high levels of fat, which may cause excessive fat deposition in visceral WAT, but it is not known how this affects fish health. Some studies have revealed the capacity of the AT to secrete immune factors in farmed animals using transcriptome analysis [14]. In addition, immune response of the adipose tissue can be modulated through the fat content in the diet in rainbow trout [15], indicating that adipose tissue should be considered as an important immune organ in fish, capable of participating in and

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influencing immune responses. However, little is known about the relationship between adipocyte or preadipocyte and inflammation in fish.

Grass carp (*Ctenopharyngodon idella*), a herbivorous freshwater fish, is an important farmed fish in China for its delicious meat and high market value [16]. It is considered as a good model for the study of lipid metabolism because grass carp store excess fat in visceral adipose tissue. In this study, we identified the immune pathways throughout adipogenesis by transcriptome analysis and compared the immune responses of adipocytes and preadipocytes under lipopolysaccharide exposure. We demonstrate that inflammatory signaling pathway is activated during grass carp adipocyte differentiation, and adipocytes produced higher levels of proinflammatory cytokines compared with preadipocytes in LPS-induced inflammation.

2. Materials and methods

2.1. Transcriptome data and identification of DEGs

The raw reads of the preadipocytes and adipocytes obtained from Liu et al., 2018 [17], and then were assembled using the Trinity (http://trinityrnaseq.sourceforge.net/) software [18]. Contigs were obtained by extending based on the overlap between sequences and then the resultant contigs were joined into transcripts with the paired-end information. To annotate the transcriptome, unigenes were searched against Nr, Nt, Swissprot, and TrEMBL using BLAST with a cut-off e-value of 0.00001. The preadipocytes and adipocytes were defined as T3 and T5, respectively.

2.2. Functional annotation and pathway enrichment analysis of DEGs

Gene expression levels were measured through short reads mapping in Reads Per Kb per Million reads (RPKM) [19]. DEGs were detected by IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6) with a general chi square test based on RPKM values among the four samples. The test results were corrected by false discovery rate (FDR). Genes were regarded as differentially expressed when the FDR < 0.001 and the absolute value of the log2 ratio > 1. GO functional analysis and KEGG pathway analysis were then carried out in differentially expressed genes. Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp) was used to annotate input genes, classify gene functions, identify gene conversions, and carry out GO term analysis and KEGG pathway, while specifying an enrichment P-value,0.05 for statistical significance.

2.3. Adipocyte isolation and treatments

Experimental grass carp were obtained from the local fish farm (Kang Le Farmer's Market). The grass carp preadipocytes were cultured as described by Ref. [20] with minor modifications. Briefly, the adipose tissue (~ 180 g) was isolated by sterile dissection from the abdominal cavity of 4–5 fishes. The tissue was washed three times with phosphate-buffered saline (PBS, pH 7.4) and minced in 0.1% Type I collagenase (Sigma, USA) with 2% BSA (Sigma) at room temperature for 30 min. The cell suspension was filtered through a 200-μm nylon filter and centrifuged at 590 g for 10 min. The sedimented cell pellet was incubated in erythrocyte lysing buffer for 10 min at room temperature, washed twice and resuspended in growth medium (GM), composed of Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. The resuspended cells were seeded in gelatin pre-coated plates at a density of approximately 10 g tissue/25 cm^2. To reach 80–90% confluency, the cells were incubated at 28 °C with 5% CO_2 for one week. The differentiation was induced in adipogenic medium (AM) containing GM supplemented with 10 μg/mL insulin (bovine) (Yangling Xin Yi Biology Technology Co., Ltd.), 10 nmol/L triiodothyronine, 1 μmol/L dexamethasone and 0.5 mmol/L 3-Isobutyl-1-Methylxanthine (IBMX). The medium was changed every 2 days. More than 90% of the cells became adipocytes at day 8 after differentiation. Cells were harvested at day 0, 2, 4, 6, and 8 after differentiation to determine gene expression during adipogenesis. LPS was obtained from Sigma-Aldrich. At day 8 after differentiation, LPS (200 ng/mL) was added to the corresponding plates. After incubation for 24 h, cells were harvested. The adipocytes were collected at 24 h. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Three replicate samples were analyzed for each treatment group. Three independent experiments were performed for each treatment and control. The gene expression levels were determined by quantitative Real-Time PCR.

2.4. RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol Reagent (TaKaRa, Otsu, Shiga, Japan) according to the instructions of the manufacturer. The purity and the concentration of total RNA were measured by a spectrophotometer at 260 and 280 nm. The integrity was tested by electrophoresis in 1.5% agarose gels. The extracted and purified total RNA was treated with RNase-free DNase to prevent the genomic DNA contamination. One microgram of total RNA was used for reverse transcription in a 10 μL reaction volume with First Strand cDNA Synthesis Kit (ToYoBo, Tokyo, Japan). Real-time RT-PCR was performed using a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The amplification was performed in a final volume of 20 μL containing 0.6 μL of each primer (0.5 μM), 1 μL of the diluted cDNA (10-fold), 10 μL of 29 SYBR®Premix Ex TaqTMII (TaKaRa, Dalian, Liaoning, China) and 7.8 μL of sterilized double-distilled water. The real-time PCR contained an initial activation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. The standard curve of each gene expression was plotted using the results of the serial dilution standards. Primer efficiency was in the range of 0.90–1. Gene expression levels were normalized to β-actin mRNA levels by calculating delta cycle thresholds (ΔCt) (ΔCt = Ct of the target gene – Ct of β-actin). Relative mRNA expression for each gene was normalized to control group by using $2^{-\Delta\Delta Ct}$ method [21].

2.5. Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) or the two-tailed Student's t -test. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Identification of differentially expressed genes

The transcriptome datasets of preadipocytes and adipocytes samples were compared using DEG. Specified criteria, the false discovery rate (FDR) < 0.001 and the absolute value of the log2 ratio > 1 were met for 5366 genes, including 4585 and 781 genes that were upregulated and downregulated, respectively (Fig. 1).

3.2. GO term enrichment analysis of DEGs

GO term enrichment analysis results varied from GO classification and expression change of DEGs (Fig. 2). For molecular function, the DEGs significantly enriched in ATP binding, metal ion binding, zinc ion binding, GTP binding, DNA binding, protein binding, calcium ion binding, nucleotide binding, nucleic acid binding, protein serine/threonine kinase activity. About cellular component, the DEGs significantly enriched in nucleus, cytoplasm, integral to membrane, plasma membrane, intracellular, membrane, cytosol, mitochondrion, Golgi apparatus, endoplasmic reticulum. As to biological process, the

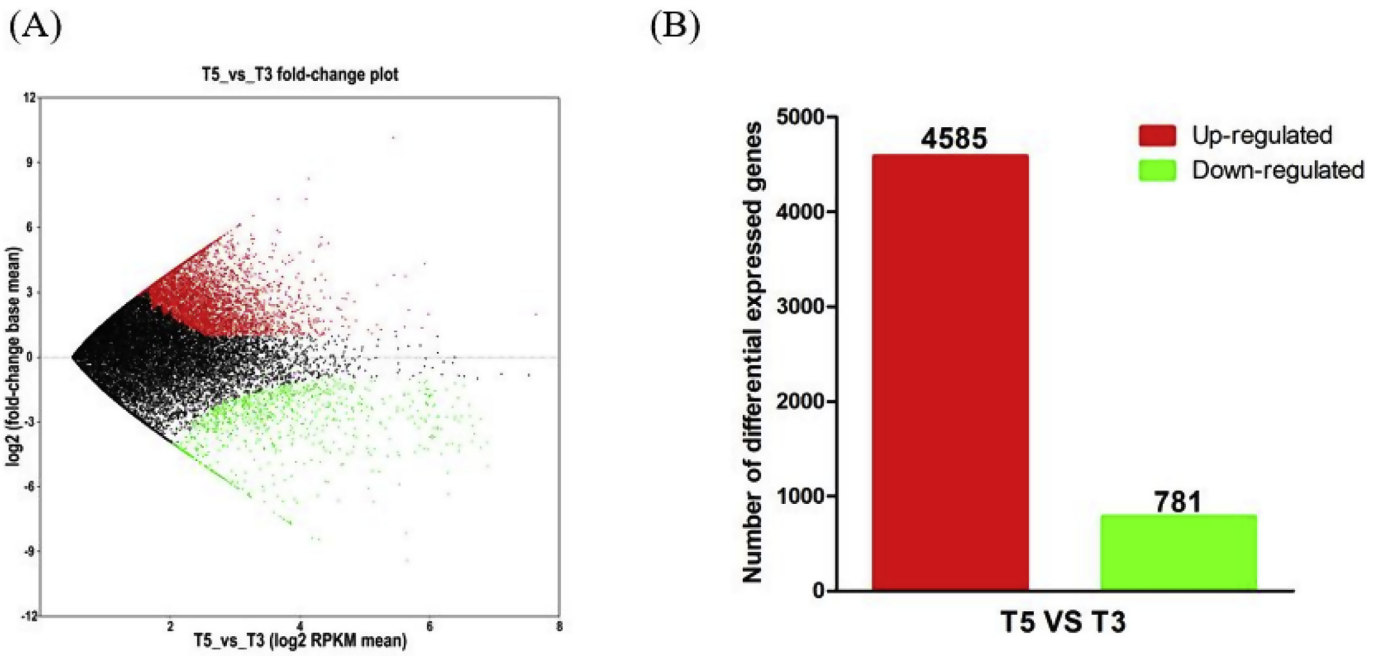

Fig. 1. Scatter plots (A) and number (B) of differential expressed analysis of unigenes by DGE. Differentially expressed genes (DEGs) were detected by IDEG6 software with a general chi square test based on RPKM values. Genes were regarded as differentially expressed when the false discovery rate (FDR) $<$ 0.001 and the absolute value of the log2 ratio $>$ 1. Red columns or dots means the unigene up-regulated, green columns or dots means the unigene down-regulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

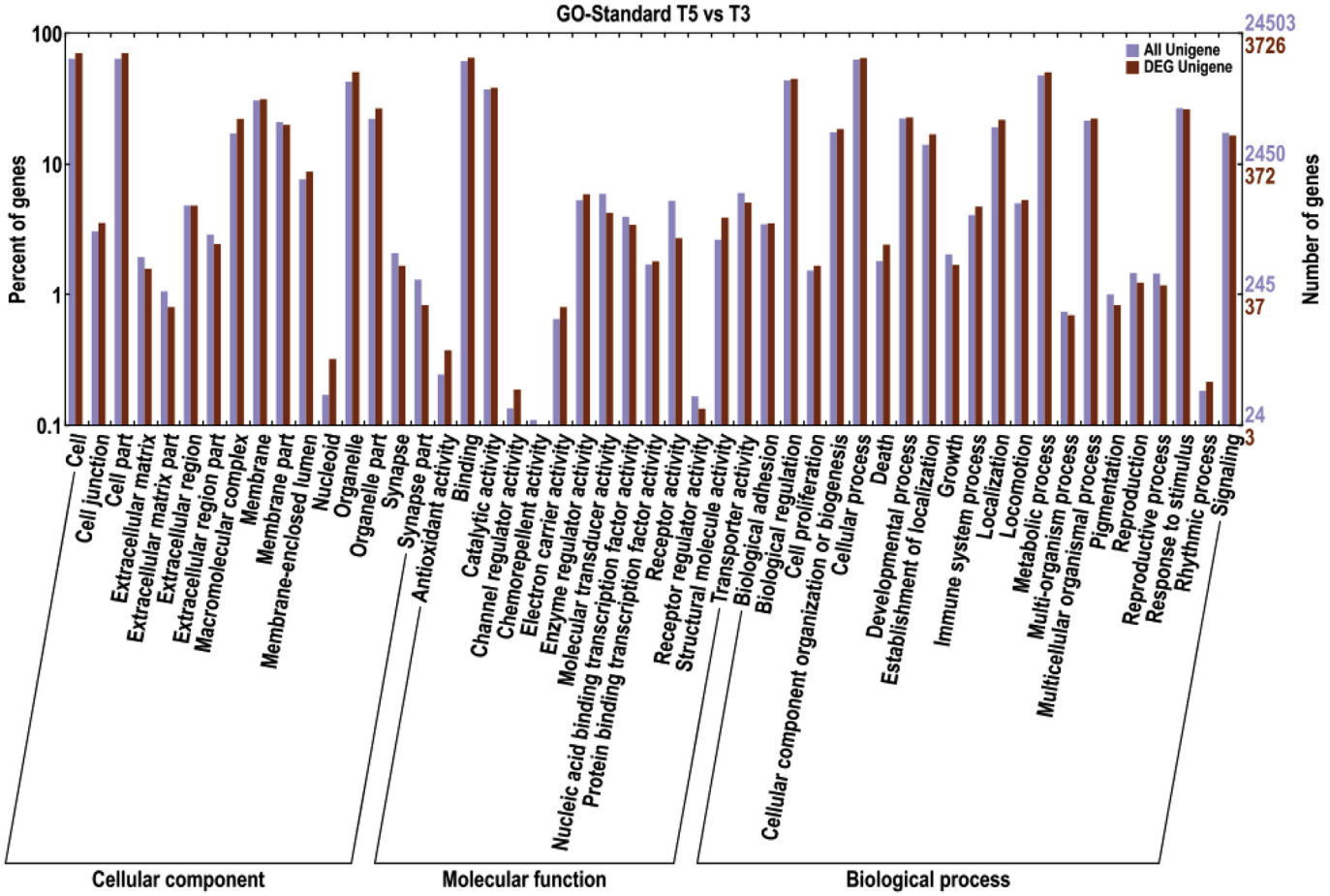

Fig. 2. Functional annotation of the DEGs based on GO categorization. The left y-axis indicates the percentage of a specific category of DEGs in that main category. The right y-axis indicates the number of genes in a category.

Table 1
The most represented GO term associated with immune response.

GO ID	Term	Count
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	24
GO:0006955	immune response	17
GO:0032088	negative regulation of NF-kappaB transcription factor activity	8
GO:0002376	immune system process	7
GO:0034138	toll-like receptor 3 signaling pathway	7
GO:0034134	toll-like receptor 2 signaling pathway	7
GO:0034142	toll-like receptor 4 signaling pathway	7
GO:0002755	MyD88-dependent toll-like receptor signaling pathway	7
GO:0035419	activation of MAPK activity involved in innate immune response	6
GO:0051092	positive regulation of NF-kappaB transcription factor activity	6
GO:0034146	toll-like receptor 5 signaling pathway	6
GO:0035682	toll-like receptor 21 signaling pathway	6
GO:0035681	toll-like receptor 15 signaling pathway	6
GO:0034154	toll-like receptor 7 signaling pathway	6
GO:0002683	negative regulation of immune system process	5
GO:0002218	activation of innate immune response	5
GO:0045087	innate immune response	4
GO:0007250	activation of NF-kappaB-inducing kinase activity	4
GO:0002682	regulation of immune system process	3
GO:0045088	regulation of innate immune response	3
GO:0050776	regulation of immune response	3
GO:0007252	I-kappaB phosphorylation	3
GO:0002224	toll-like receptor signaling pathway	3

DEGs significantly enriched in oxidation-reduction process, positive regulation of transcription from RNA polymerase II promoter, GTP catabolic process, protein transport, signal transduction, small GTPase mediated signal transduction, translation, regulation of cellular process, intracellular protein transport, phosphorylation.

A total of 999 unigenes involved in immune system process, and 176 of them were differential expression genes (Fig. 2). More detailed GO enrichment associated with immune response analysis results are shown in Table 1. The results showed that NFκB signaling pathway was activated during adipogenesis.

3.3. KEGG pathway enrichment analysis of DEGs

On KEGG pathway analysis, the DEGs were significantly enriched in Focal adhesion, MAPK signaling pathway and Regulation of actin cytoskeleton (Table 2). As shown in Table 2, many pathways involved in the immune response were found, such as Jak-STAT signaling pathway, Toll-like receptor signaling pathway (Suppl. Fig. 1), Cytokine-cytokine receptor interaction, Adipocytokine signaling pathway (Suppl. Fig. 2), Chemokine signaling pathway and T cell receptor signaling pathway.

3.4. Verification of DEGs by RT-PCR

Given that altered adipokine secretion is one of characteristics of chronic inflammation in adipose tissue, we selected the DEGs involved in adipocytokine signaling pathway to verify the transcriptome sequencing results using Real-time RT-PCR (Table 3). The gene expression levels of NF-kB, TNFR1, TNFR2, JNK, LEPR, AdipoR were enhanced significantly with the development of preadipocyte and reached maximum levels by day 8 compared with other days after differentiation. ($P < 0.05$). (Fig. 3).

3.5. LPS induced inflammation in grass carp preadipocytes and adipocytes

To investigate whether preadipocytes and adipocytes have immune response, we detected the expression of inflammatory markers, including NF-kB, IL-6, MCP-1 and TNFα, after LPS treatment. These inflammatory markers were higher expressed in adipocytes or preadipocytes under LPS exposure than control (Fig. 4).

Table 2
The most represented KEGG pathways in the unigene data set.

Pathway	DEGs with pathway annotation	p_value
Focal adhesion	115	5.56E-05
MAPK signaling pathway	109	3.22E-04
Regulation of actin cytoskeleton	105	3.76E-05
Oxidative phosphorylation	69	1.52E-08
Insulin signaling pathway	69	1.29E-03
Protein processing in endoplasmic reticulum	67	9.13E-04
Wnt signaling pathway	62	4.04E-01
TGF-beta signaling pathway	50	7.53E-05
ErbB signaling pathway	49	6.54E-04
Jak-STAT signaling pathway	47	7.62E-04
GnRH signaling pathway	46	1.78E-01
VEGF signaling pathway	42	6.19E-04
Calcium signaling pathway	42	9.99E-01
Toll-like receptor signaling pathway	33	9.70E-03
Cytokine-cytokine receptor interaction	33	8.13E-01
Adipocytokine signaling pathway	32	4.18E-02
mTOR signaling pathway	29	3.02E-02
Glycerophospholipid metabolism	26	8.06E-01
Citrate cycle (TCA cycle)	20	1.94E-01
p53 signaling pathway	19	5.20E-01
Notch signaling pathway	19	9.74E-01
Glycolysis/Gluconeogenesis	17	9.93E-01
PPAR signaling pathway	14	9.16E-01
Peroxisome	14	9.48E-01
Glycerolipid metabolism	13	9.32E-01
Chemokine signaling pathway	7	6.14E-01
Fatty acid metabolism	7	9.94E-01
T cell receptor signaling pathway	6	2.61E-01

Table 3
Specific Primers used for qPCR in this study.

Primers	Sequences (5′-3′)
TNFR1 F	AGAGATCAGATTGTGGTTGCAG
TNFR1 R	GTTGGATCTGTCGTGGTGC
TNFR2 F	AGGACCGAGTAGTGTCTGGA
TNFR2 R	TGTGGTTTTGCTTGCTGTCG
JNK F	CGGCTGACGGTGTTTAATGTG
JNK R	TCCCACTGAGTGCCGTGA
NF-κB F	GAAGAAGGATGTGGGAGATG
NF-κB R	TGTTGTCGTAGATGGGCTGAG
TNFα F	GCTGCTGTCTGCTTCACGCT
TNFα R	TGCCTGGTCCTGGTTCACTCT
IL-6 F	CCCTGGTCAACGACATCAA
IL-6 R	GTCCACCCTTCCTCTTGCT
MCP1 F	GACTGTCCCAGACGAGCTAT
MCP1 R	CTTTAGCAACATGTCCGCTCG
LEPR F	GAACGGGCAGGTGAAGAT
LEPR R	ACCCTGGTTCGTCTACTTTC
Adipo R F	TAGTGCAGAGAAATCCAGAG
Adipo R R	GAAGCAGGTAATCATTGTCC
β-actin-F	TCCACCTTCCAGCAGATGTGGATT
β-actin-R	AGTTTGAGTCGGCGTGAAGTGGTA

3.6. Adipocytes produced higher levels of proinflammatory cytokines compared with preadipocytes in LPS-induced inflammation

To compare the inflammatory response of preadipocytes and adipocytes in LPS-induced inflammation, we detected the expression of NF-kB, IL-6, MCP-1 and TNFα. These inflammatory markers were higher expressed in adipocytes compared with preadipocytes under LPS exposure (Fig. 5).

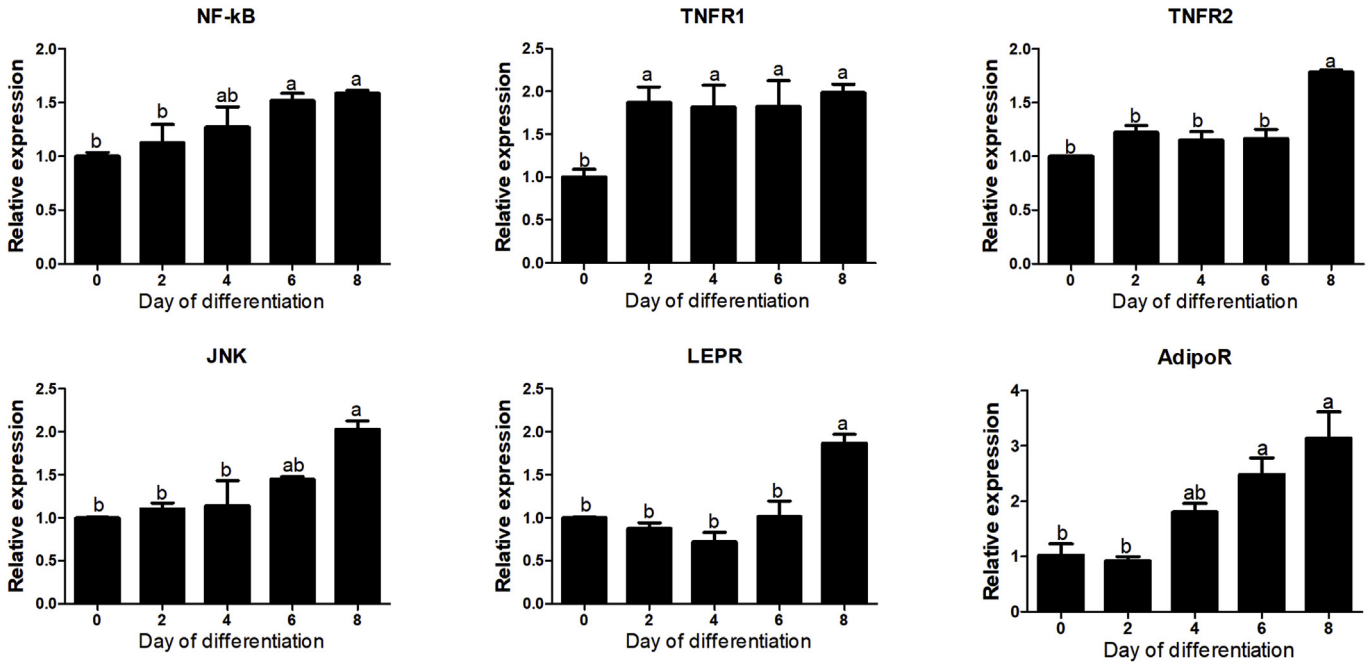

Fig. 3. Validation of differentially expressed genes involved adipocytokine signaling pathway during adipogenesis with Real-time RT-PCR on days 0, 2, 4, 6, and 8 of differentiation. Gene expression was normalized to β-actin and was referred to the expression of genes in day 0 (D0). Data are means ± SEM, n = 3. Different letters indicate significant differences at $P < 0.05$.

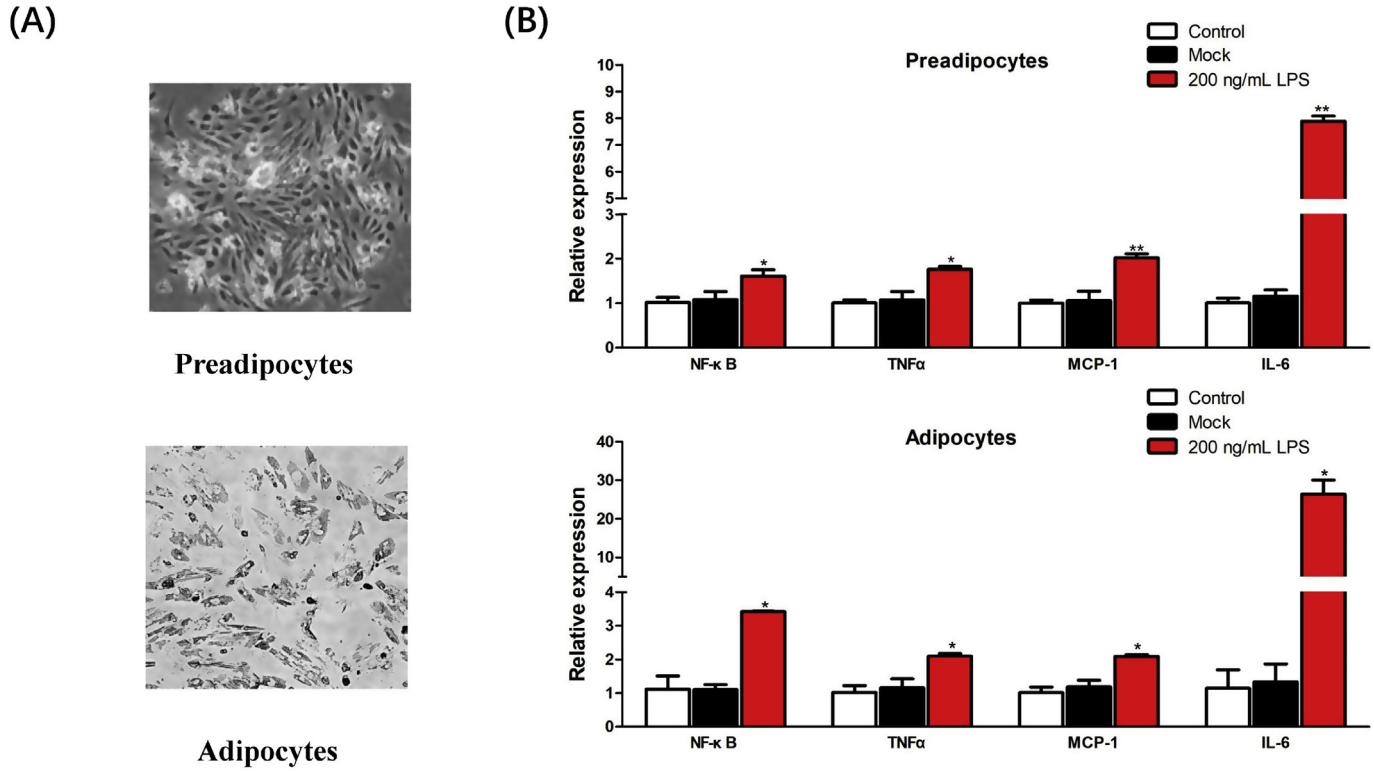

Fig. 4. LPS induced the expression of inflammatory markers, NF-kB, IL-6, MCP-1 and TNFα, in adipocytes and preadipocytes. Data (mean ± SEM, n = 3) were normalized to housekeeping gene (β-actin). *$P < 0.05$, **$P < 0.01$ indicated a significant difference as determined by Student's t-test.

4. Discussion

Excessive body fat is considered as a chronic inflammatory disorder. Adipose tissue inflammation mediates the association between excessive body fat accumulation and the chronic inflammatory diseases [22]. In this study, we characterized transcriptome changes during adipogenesis using bioinformatics analytical tools. We found that 1) inflammatory signaling pathway may be activated during grass carp adipocyte differentiation; 2) Adipocytes have greater potency compared with preadipocytes in LPS-induced inflammation. These results would promote an understanding of the role of adipocytes and preadipocytes in immune.

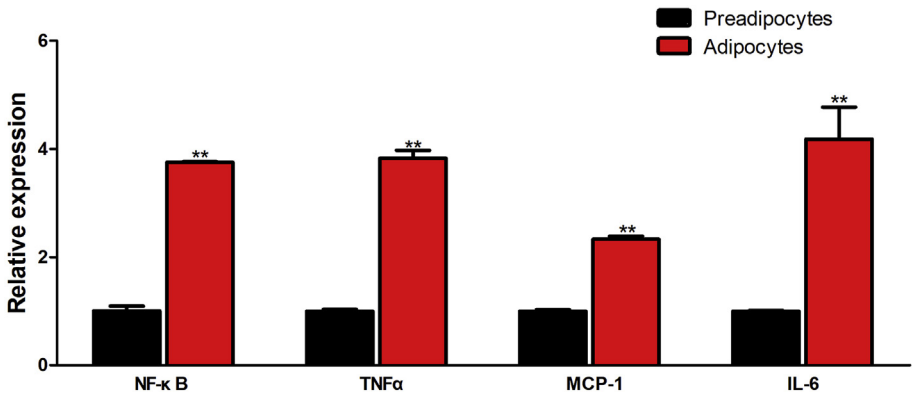

Fig. 5. The inflammatory markers, NF-kB, IL-6, MCP-1 and TNFα, were higher expressed in adipocytes compared with preadipocytes under LPS exposure. Preadipocytes and adipocytes (at day 8 after differentiation) within the same batch cell were treated with LPS (200 ng/mL) for 24 h. The gene expression levels were determined by quantitative Real-Time PCR. Data (mean ± SEM, n = 3) were referred to the control treatment using β-actin as a control. $^{**}P < 0.01$ indicated a significant difference as determined by Student's t-test. Legends to supplementary files.

White adipose tissue (WAT) in mammals is not only an organ for energy storage, but also now recognized as a highly active endocrine tissue producing numerous secretory proteins, including adipokines, such as IL-6, MCP-1 and TNFα [22,23]. In our study, the mRNA levels of these adipokines were increased in preadipocytes and adipocytes under LPS exposure, indicating that preadipocytes and adipocytes are both involved in endocrine functions of adipose tissue in fish. Adipokines regulate adipose tissue structure, inflammation and metabolism in autocrine and paracrine manners [24]. For example, IL-6 is a paracrine regulator of adipose tissue by reducing the gene expression of several adipose differentiation markers [25]. We found that the adipocytokine signaling pathway was activated during adipogenesis. These results suggest that grass carp preadipocytes and adipocytes may affect WAT function in an autocrine or paracrine manner. Challa et al. [26] identified that adipokines regulated adipocyte differentiation through positive or negative paracrine and autocrine feedback loop mechanisms. Hence, future study should focus on the paracrine, autocrine and crosstalk of adipocytes and preadipocytes effects on *de novo* grass carp adipocyte differentiation.

Increased production of adipokines is involved in adipocyte inflammation, leading to the chronic low-grade inflammatory state [22]. Hence, adipose tissue is increasingly seen as playing an important role in immune function [14–16]. It is reported that NF-kB mediates these pro-inflammatory responses [27]. In this study, many biological process involved NFκB signaling pathway were activated and the level of NFκB was increased during adipogenesis. Given that NF-kB activation is at the core of many proinflammatory transcriptional programs [28], adipocyte should be considered as an inflammatory cell of systemic importance in fish, besides acting primarily as a metabolic cell, thus providing further evidence of the immune role of WAT in phylogenetically remote vertebrate classes [29]. In addition, Toll-like receptor signaling pathway was also activated during adipogenesis in this study. Toll-like receptors (TLRs) play a key role in innate immune responses [30]. These results demonstrate that adipocyte differentiation may play a dynamic role in the regulation of inflammation and innate immunity in fish. Therefore, limiting WAT expansion by inhibiting adipocyte differentiation and reducing inflammation may be effective in preventing the progression of excessive fat and the development of associated complications in farmed fish.

Increasing evidences found that preadipocytes and adipocytes from WAT both have high inflammatory response, leading the expression and secretion of inflammatory markers, such as IL-6, MCP-1 and TNF-α in mouse [9–11,13,22]. In our study, we also found that grass carp preadipocytes and adipocytes have inflammatory response, as described in Atlantic salmon [29], suggesting that the immune activity is conserved in vertebrates white adipocytes. It has been reported a greater potency of preadipocytes compared with adipocytes on inflammatory conditions such as under normoxia, hypoxia and TNFα exposure [10,31]. However, we found that adipocytes produced higher levels of proinflammatory cytokines compared with preadipocytes in LPS-induced inflammation. These results indicated that different expression of adipokines of preadipocytes and adipocytes contribute to their different inflammatory response in various conditions.

In conclusion, we identified the biological process and pathway that are related to immune activity during adipogenesis on the basis of bioinformatics analysis of DEGs, thus providing further evidence of the immune role of adipocytes in phylogenetically remote vertebrate classes. Results from the present study revealed that NFκB signaling pathway, Toll-like receptor signaling pathway and Adipocytokine signaling pathway, may play a crucial role in grass carp adipocyte differentiation. Further study found that preadipocytes and adipocytes both have immune response, and have different immune response in LPS-induced inflammation. Future work in this area should address the contribution of preadipocytes and adipocytes on inflammatory in farmed fish that suffered excessive fat deposition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.295.

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