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The fat accumulation promotion effects of dihydrxytetraphenylmethane and its underlying mechanisms via transcriptome analysis

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ABSTRACT

Dihydrxytetraphenylmethane, also known as Bisphenol BP (BPBP), has been increasingly used in industrial production and more frequently detected in the environment as an alternative plasticizer of BPA. However, there are no reports about BPBP in food safety or its effects on cellular lipogenesis. The purpose of this research was to investigate the influence and potential mechanisms of BPBP on adipogenesis in 3T3-L1 cells. Cells were treated with 4 concentrations (0.01, 0.1, 1, and 10 μM) of BPBP and the results showed that treatment with at low concentrations (0.01 μM) promoted cell fat differentiation and triglyceride accumulation. RNA-seq data showed that a total of 370 differentially expressed genes between control and the low-dose BPBP-treated group were determined, including 227 upregulated genes and 143 downregulated genes. Some key genes related to adipocyte differentiation and adipogenesis were significantly enriched after BPBP treatment, including *PPAR-γ*, *Adipoq*, *Nr1h3* and *Plin1*. Pathway analyses suggest that the activation of *PPAR-γ* signaling pathway may be key for BPBP to promote adipocyte differentiation and fat accumulation. Our work provides evidence for the potential obesogenic effect of BPBP and may call for further research on the safety of the chemical in food products.

1. Introduction

Food safety issues have received widespread attention, among which chemical contamination is a very concerning food safety hazard. People's concerns about the food chemical contamination are often focused on the food itself, such as the abuse of food additives and pesticide residues. However, food contamination by chemicals migrating from plastic food packaging is often overlooked. In fact, as the hazards arising from this mode of food chemical contamination often take a long time to manifest and are not easily detected, their impact on human health may be more serious.

Nowadays plastics is widely used in all aspects of people's daily life, especially in food fields such as food packaging, food containers or plastic wrap. These plastic products often use some Endocrine disrupting chemicals (EDCs), which are defined as a kind of exogenous substances that interfere with the human endocrine system (Street et al., 2018), as additives to improve performance or reduce cost during production. And the EDCs contained in these plastic products may penetrate into food, water and the environment under some conditions, such as heating or prolonged soaking and eventually enter the human body, causing harm to the body's endocrine system and increasing the risk of developing diseases (Ong et al., 2022). These low-dose EDCs derived from food plastic packaging may accumulate in humans for a long time and cause chronic poisoning. And the effects on human health of exposure of this

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magnitude may take a long time to manifest, so the threat to human health and food safety may be greater (Almeida et al., 2018; Grob et al., 2006; Ong et al., 2022). Therefore, it is of practical significance to explore the effects of these EDCs on the body or cells to ensure food safety.

Bisphenol compounds (BPs) are typical EDCs characterized by the presence of two phenyl hydroxyl groups. Bisphenol A is commonly used in the industrial manufacture of polymeric materials such as polycarbonates and epoxy resins (Almeida et al., 2018; Oliviero et al., 2022). An accumulating body of evidences have exhibited that BPA may be promoted adipogenesis, and increased cellular triglyceride content and elevated gene expression of some adipogenic transcription factors in vitro, and associated with some metabolic disorders, such as obesity and type 2 diabetes (Hwang et al., 2018; Lang et al., 2008). (Choi et al., 2021; Masuno et al., 2002; Sargis et al., 2010). These studies have also led to BPA being banned or restricted in food areas in many countries (Baluka and Rumbeiha, 2016). Therefore, some BPA analogues were produced as substitutes for BPA, such as Bisphenol S (BPS), Bisphenol F (BPF), Bisphenol B (BPB), Bisphenol AF (BPAF) and Bisphenol BP (BPBP). However, because these analogues have similar chemical structures with bisphenol A, its safety was also widely discussed. Previous studies have shown that BPS has similar effects of promoting adipogenesis as BPA(Ahn et al., 2020; Reina-Pérez et al., 2021). Other studies have shown that BPB, BPF, BPAF can disrupt adipocyte metabolism and insulin signaling by inhibiting the PPAR pathway (Schaffert et al., 2021).

Dihydrxytetraphenylmethane, also known as Bisphenol BP (BPBP), is a novel bisphenol compound that has also been used as an alternative to BPA. Because of the similar structure with BPA, it has the potential of being an EDC. However, there are no reports on the safety of BPBP in food, nor on its effects on the body or cells (e.g., adipogenesis and lipid metabolism). Therefore, to fill this gap and provide a reference for food safety studies of BPBP, we utilized a cellular model combined with transcriptomic analysis to investigate the influence and mechanisms of BPBP on adipogenesis.

2. Materials and methods

2.1. Materials

Calf serum (CS), fetal calf serum (FBS), Dulbecco modified Eagle medium (DMEM), Revert-Aid First Strand cDNA Synthesis Kits and Trizol reagent were purchased from Thermo Fisher Scientific Co., Ltd (Rockford, IL). Insulin, dexamethasone and isobutyl methyl xanthine (IBMX) were gained from Sigma-Aldrich Company (St. Louis, Missouri, USA). SYBR® Green Master Mix was gained from TIANGEN BIOTECH (Beijing, China). 3T3-L1 cells were provided by Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

2.2. 3T3-L1 cells culture and triglyceride (TG) determination

3T3L1 cells were cultured according to our previous description (Qi et al., 2022). Adipocyte differentiation was activated using different mixed media in 3 stages. Two days after confluency (Day 0): IBMX (0.5 mM), insulin (1 μg/mL), dexamethasone (0.1 μM), and DMEM with FBS (10%); On day 2: insulin (1 μg/ml), dexamethasone (0.1 μM), and DMEM with FBS (10%); Starting on day 4: DMEM with 10% FBS. From day 0, cells were treated with BPBP dissolved in DMSO (Concentrations of 0.01, 0.1, 1, and 10 μM, respectively) and 0.02% DMSO (as control).

Cells were washed twice after 8 days of differentiation before harvest. The content of triglycerides (TG) was quantified using the Triglycerides (TG)Assay Kit (GPO-PAP Method), provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China) according to the instructions.

2.3. Reverse transcription-quantitative PCR (RT-qPCR)

3T3L-1 cells differentiated for 24 h, 48 h, 96 h and 8 d were completely digested with Trizol reagent and collected. Total RNA of the samples was extracted without RNase and cDNA libraries were synthesized using the Revert-Aid First Strand cDNA Synthesis Kits as previously reported (Chen et al., 2022; Qi et al., 2022). The RT-qPCR was then conducted on a StepOne Plus real-time PCR system. The reaction system was formulated according to the kit instructions. The primer sequence and reaction conditions for RT-qPCR are shown in Table S2 and Table S3. GAPDH was used as an internal reference gene.

2.4. RNA extraction, library construction and sequencing

Cell samples were stringently collected and snap frozen in liquid nitrogen before being sent to Gene Denovo Biotechnology CO. (Guangzhou, China) for subsequent RNA extraction, purification, and sequencing. Briefly, extracted high-quality total RNAs were enriched for eukaryotic mRNAs by Oligo (dT). Products were cut into small fragments and reverse transcribed into cDNA. Purified cDNAs were amplified by PCR after modification and ligation reactions. The cDNA library was then sequenced using Illumina Novaseq 6000. The raw data were quality controlled for clean data and further used for bioinformatics analysis.

2.5. Bioinformatics analysis

Differentially expression genes (DEGs) between the comparison groups were analyzed by DESeq2 software. And the DEGs screening criteria was as follows: $|\text{Fold change}| > 1.2$ and p-value <0.05.

Gene Ontology (go) is a standardized classification system describing gene function. The number of DEGs localized in different GO terms was calculated, and significant enrichment was defined by hypergeometric test (p-value <0.05).

Kyoto Encyclopedia of Genes and Genomes (KEGG) was the main pathway related database. Pathway significant enrichment analysis was performed in units of KEGG pathway, and a hypergeometric test was applied to search for pathways whose genes were significantly enriched relative to the genome-wide background (p-value <0.05).

2.6. Statistical analysis

Three biological replicates were used for triglyceride assay and RNA-seq analysis. And three biological replicates with three technical replicates were used for RT-qPCR. Data were exhibited as the mean ± SEM and analyzed using GraphPad Prism version 9.4. One way ANOVA followed by Tukey's multiple comparison tests was performed to analyze the differences. The p-value $<$ 0.05 was considered statistically significant.

3. Results

3.1. Bisphenol BP increased triglyceride accumulation in 3T3-L1 cells

3T3-L1 cells were treated with 4 different concentrations (0.01 μM, 0.1 μM, 1 μM, and 10 μM) of bisphenol BP (BPBP) to investigate the effect of BPBP on the adipogenic capacity of cells. Interestingly, the effect of BPA treatment on cells was not shown to be concentration-dependent. As shown in Fig. 1, neither 0.1 μM nor 1 μM BPBP treatment group exhibited significant differences from the control group. However, compared to the control group, although BPBP treatment at 0.01 μM and 10 μM both significantly increased the content of triglyceride in 3T3-L1 cells, treatment at 10 μM was higher than 0.01 μM. This result indicates that BPBP treatment at 0.01 μM and 10 μM both exhibits a promoting effect on fat accumulation in 3T3-L1 cells.

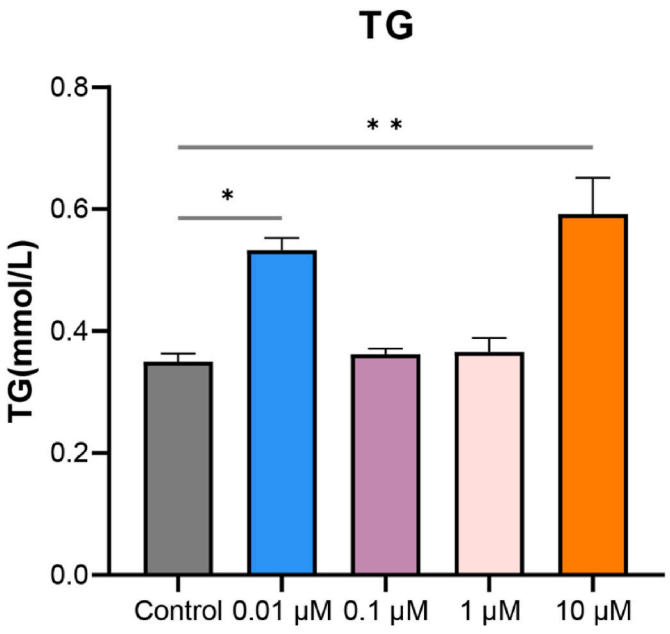

Fig. 1. The content of triglyceride in 3T3L-1 cells treated with different concentrations of BPBP. Cells were treated with BPBP for 8 days. Numbers are mean ± SEM (n = 3). *, $p < 0.05$, **, $p < 0.01$.

3.2. Effects of BPBP on the expression of adipogenic regulatory factors

To further explore the effects of BPBP on adipocyte differentiation, the expression of 5 adipogenic regulators (Fatty acid synthetase, *FAS*; fibroblast growth factor 10, *Fgf10*; peroxisome proliferator-activated receptor γ, *PPAR-γ*; snail family transcriptional repressor 2, *SNAI2*; and stearyl coenzyme A dehydrogenase-1, *SCD1*) at different treatment times (24 h, 48 h, 96 h and 8 days) and treatment concentrations (0.01 μM and 10 μM) were determined by RT-qPCR.

As shown in Fig. 2, after 24 h of BPBP treatment, there were no significant differences in the expression of any of the genes treated with 0.01 μM BPBP, except for the expression of *FAS*, which was significantly exceeded than that of the control. Nevertheless, the expression of *PPAR-γ*, *SNAI2* and *SCD1* were significantly decreased in the 10 μM BPBP group. Besides, after 48 h and 8 days of BPBP treatment, the expression of most genes was not significantly different from that of the control group, except for the expression of individual genes, which was down-regulated in the two different concentrations of BPBP treatment. Most notably, after 96 h of 0.01 μM BPBP treatment, the expression of *Fgf10*, *PPAR-γ* and *SNAI2* were significantly upregulated compared with control group. Moreover, the expression of *Fgf10* and *PPAR-γ* in cells treated with 10 μM BPBP were also higher than control group. From the above, BPBP treatment for 96 h may be a critical period for inducing adipogenesis in 3T3-L1 cells.

Thus, combined with the available data, we finally chose to further perform transcriptomic analysis on cells treated with 0.01 μM BPBP for 96 h.

3.3. Relationship analysis of samples and identification of differentially expressed genes

As shown in Table S1, more than 40123322 raw reads obtained from sequencing machines per library. And after further filtered (including removed low-quality reads and adapter), The acquisition rate of clean reads exceeds 99.58% in each library. The high quality of the RNA-seq offline data allowed further bioinformatics analysis.

Principal Component Analysis (PCA) of the samples showed that the contribution of principal component 1 (PC1) and principal component 2 (PC2) to sample variation in distinguishing control and BPBP-treated groups was 71.4% and 17.5%, respectively (Fig. 3A). And the Pearson's correlation heatmap was shown in Fig. 3B. The BPBP treatment led

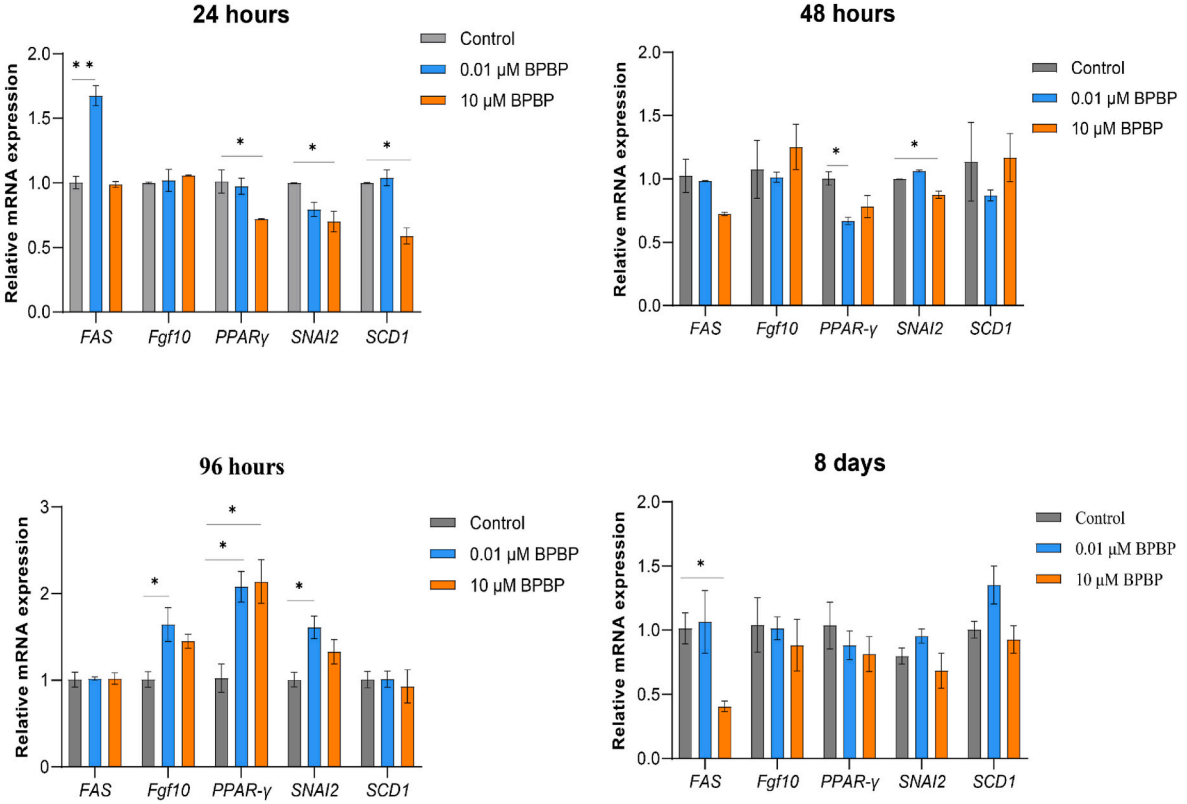

Fig. 2. Relative expression of selected genes at different BPBP treatment concentrations and treatment times. Numbers are mean ± SEM (n = 3). *,$p < 0.05$.

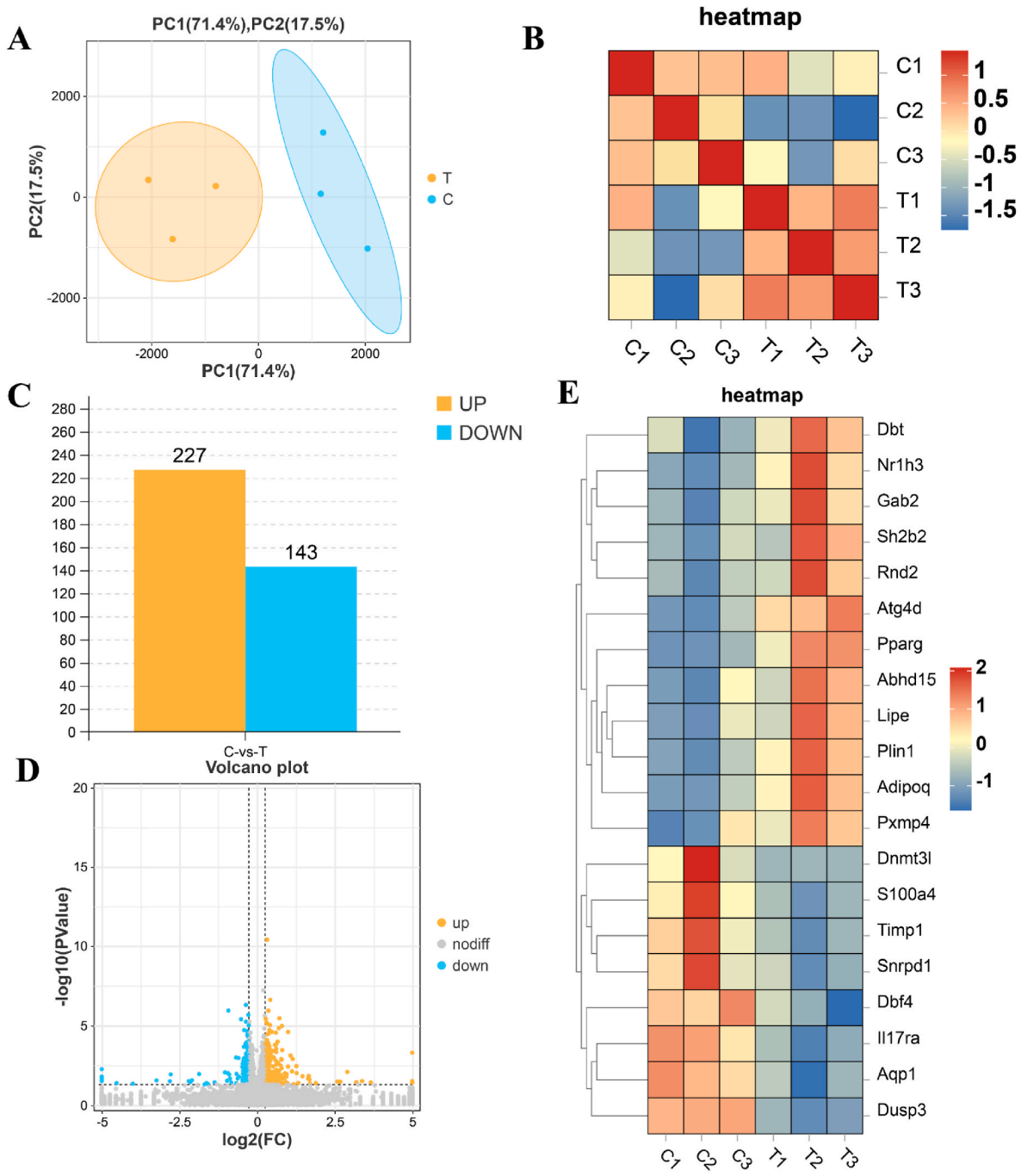

Fig. 3. Relationship analysis of samples and identification of differentially expressed genes. Principal component analysis (PCA) (A), and correlation heatmap (B) of sample relationship in C-vs-T. Holistic statistics (C), volcano plot analysis (D), and heatmap (E) of DEGs in C-vs-T. C represents three samples obtained from the medium without any treatment; T represents three samples obtained from the medium with 0.01 μM BPBP.

to a distinct clustering change of genes expression and an obvious trend of separation of genes expression was also observed between control and BPBP group.

370 DEGs between control and BPBP group were identified, including 227 upregulated genes and 143 downregulated genes (Fig. 3C). The distribution of DEGs was exhibited in Fig. 3D. The first 20 upregulated and downregulated genes are visualized in the expression heat map through Z score (Fig. 3E). The results suggested that 0.01 μM BPBP treatment significantly altered genes expression during 3T3-L1 differentiation.

3.4. GO enrichment and KEGG pathways enrichment analysis of DEGs

To further explore the main biological functions exercised by the DEGs generated by BPBP treatment of 3T3L-1 cells, GO functional enrichment analysis of DEGs was performed. 370 DEGs were assigned to the 56 level-2 functional group (Fig. 4A). In biological process category, “cellular process” was the main functional group, followed by “biological regulation”, “metabolic process” and “regulation of biological process”. The DEGs were subjected to enrichment analysis in the biological process category, and the significant enrichment bubble plot is shown in Fig. 4B. The DEGs were significantly enriched in GO term “fat cell differentiation”.

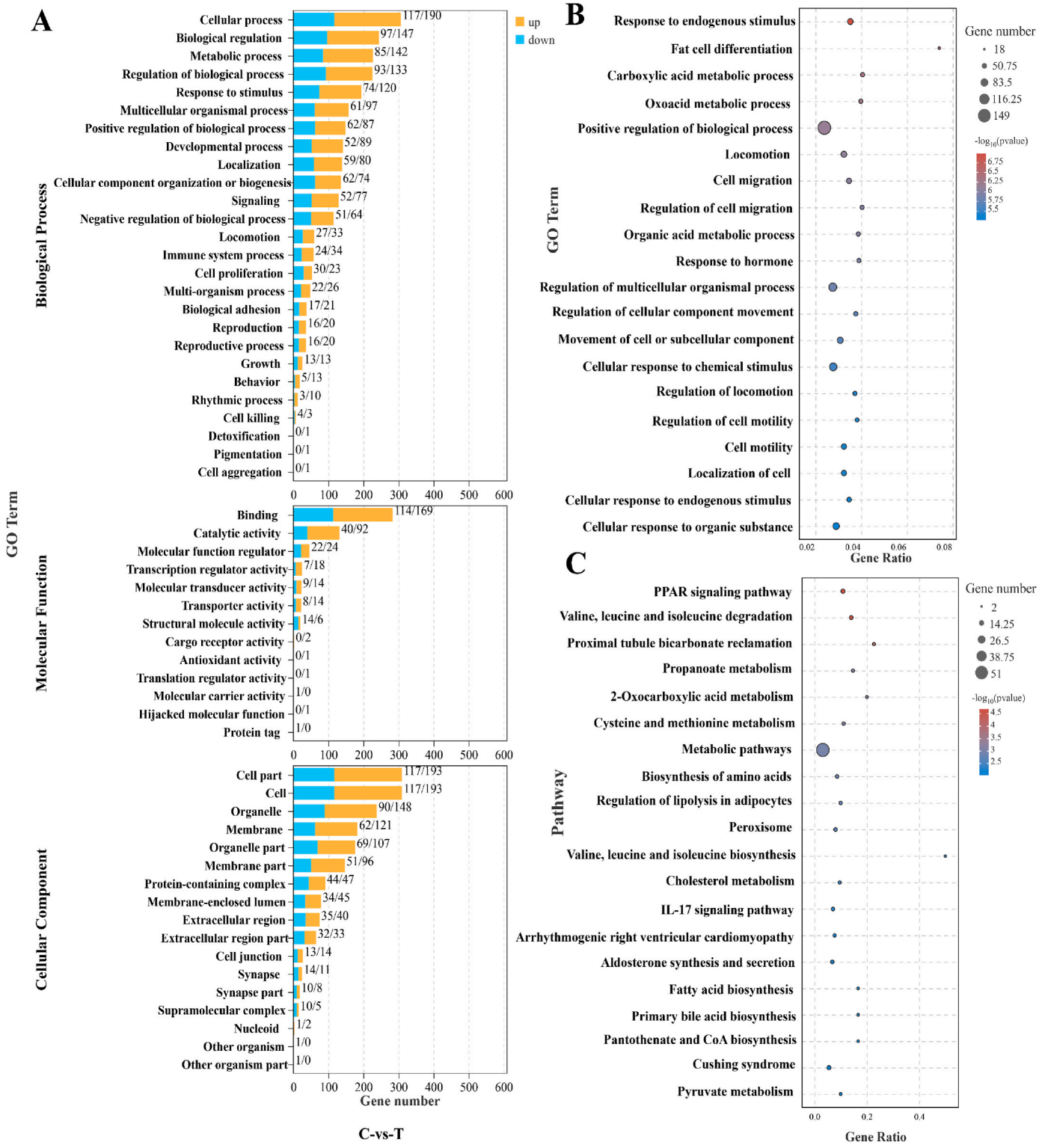

Fig. 4. GO enrichment and KEGG pathways enrichment analysis of DEGs. (A) Level-2 GO terms annotation Histogram. (B) DEG significantly enriched bubble diagram in biological process category. (C) Bubble diagram of the top 20 dominant enrichment KEGG pathway. C represents three samples obtained from the medium without any treatment; T represents three samples obtained from the medium with 0.01 μM BPBP.

To further explore the effects of BPBP on the complex biological behaviors of 3T3L-1 cells, the KEGG pathway enrichment analysis of 370 DEGs was carried out. The top 20 dominant enriched KEGG pathway were shown in Fig. 4C. The "*PPAR-γ* signaling pathway", "regulation of lipolysis in adipocytes", "*IL-17* signaling pathway" and "fatty acid biosynthesis" were significantly enriched. And *PPAR-γ* has been implicated as a key regulation factor of adipocyte differentiation and adipogenesis.

The results of GO enrichment and KEGG pathway analysis both indicated that treatment at 0.01 μM BPBP affected the complex gene regulatory network in adipocytes.

3.5. Candidate DEGs associated with the fat cell differentiation

After analyzing the DEGs enriched in GO term "fat cell differentiation" and KEGG pathways related to adipogenesis, some key genes potentially involved in BPBP affecting the fat accumulation process in 3T3L-1 cells were screened (Fig. 5). Especially, *PPAR-γ* (peroxisome proliferator activated receptor-γ), *Adipoq* (adiponectin), *Nr1h3* (nuclear receptor subfamily 1, group H, member 3) and *Plin1* (perilipin 1) were significantly up-regulated after BPBP treatment, and these genes were reported to be important regulation factors in fat cell differentiation and adipogenesis. Furthermore, the expression of *IL-17ra* were down-regulated after BPBP treatment. Previous studies have suggested that *IL-17ra* may be a key target influencing fat accumulation.

3.6. Confirmation of DEGs using RT-qPCR

In order to verify the expression level of four candidate genes (including *PPAR-γ*, *Adipoq*, *Nr1h3* and *Plin1*) related to adipocyte differentiation obtained from RNA-seq, we measured them by RT-qPCR. The RNA-seq results showed that after 96 h of 0.01 μM BPBP treatment in 3T3-L1 cells, the expression of *PPAR-γ* ($log_2FC = 0.67$), *Adipoq* (log2FC = 0.84), *Nr1h3* (log2FC = 0.42) and *Plin1* (log2FC = 0.61) was up-regulated by 1.59, 1.79, 1.34 and 1.53 times, respectively. Similar to the expression trend in the RNA-seq results, the RT-qPCR results showed that BPBP treatment also significantly increased the expression of the above genes, except for *Nr1h3* (Fig. 6).

4. Discussion

In the current study, we reveal that BPBP treatment promotes adipogenesis in 3T3L-1 cells. Our results further indicated that BPBP may promote fat accumulation by affecting a complex adipogenic regulatory network in 3T3L1 cells. Furthermore, four DEGs (*PPAR-γ*,*Adipoq, Nr1h3* and *Plin1*) enriched in GO term "fat cell differentiation" and KEGG pathways related to adipogenesis were identified, which may play

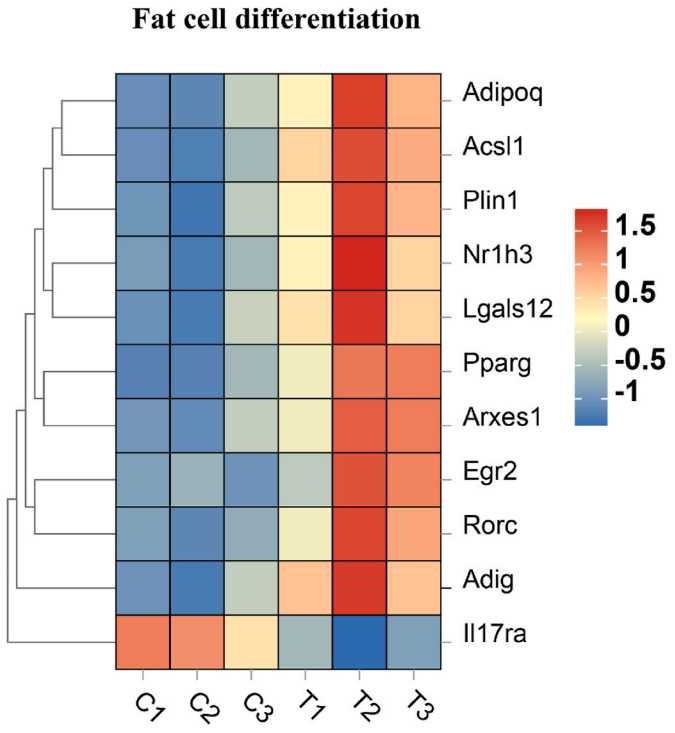

Fig. 5. Candidate DEGs associated with the fat cell differentiation. C represents three samples obtained from the medium without any treatment; T represents three samples obtained from the medium with 0.01 μM BPBP.

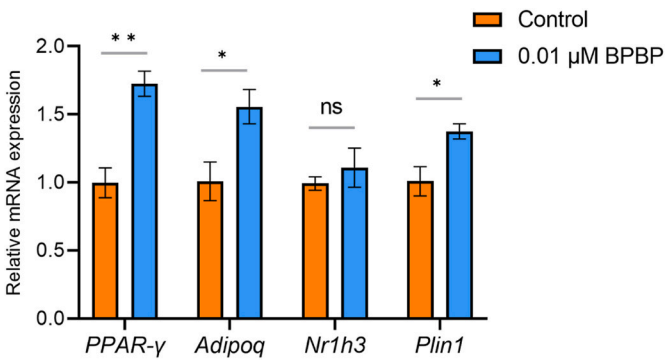

Fig. 6. Relative mRNA expression of the candidate genes by RT-qPCR. Numbers are mean ± SEM (n = 3). *, P < 0.05; **, P < 0.01.

critical roles in BPBP induced fat accumulation.

Peroxisome proliferator activated receptor-γ (*PPAR-γ*) is a nuclear hormone receptor that is highly expressed in adipocytes. *PPAR-γ is* a key transcriptional regulator involved in the differentiation and lipid metabolism of preadipocytes, whereas cells lacking *PPAR-γ* fail to differentiate into adipocytes (Brun et al., 1996; Rosen et al., 1999). The expression of *PPAR-γ* promotes adipose tissue production and decreases leptin expression, which in turn inhibits lipolysis and promotes fat accumulation (Kallen and Lazar, 1996; Picard et al., 2004). Our data exhibited that BPBP intervention significantly upregulated the expression of *PPAR-γ* and ultimately manifested as increased cellular triglyceride content. In addition, KEGG enrichment analysis revealed that *PPAR-γ* signaling pathway and some other lipid metabolism regulatory pathways were significantly enriched after BPBP treatment. These results indicate that *PPAR-γ* may be a critical target for BPBP to promote adipogenesis in 3T3L1 cells.

Perilipins are a family of proteins that coat the surface of adipocyte lipid droplets and are currently found in five isoforms, encoded by *Plin1-5*, respectively (Greenberg et al., 1991). *Plin1* is mainly expressed on the surface of lipid droplets in white and brown mature adipocytes, where it is considered as an important regulator of the lipolytic process, possibly acting as a barrier for lipases to and from the lipid droplets, protecting the lipid droplets from basal lipolysis (Blanchette-Mackie et al., 1995; Zhai et al., 2010). *Plin1* knockout mice are leaner and resistant to diet induced obesity (Krahmer et al., 2013). TNF-α decreases cellular *Plin1* levels and leads to increased cellular basal lipolysis and free fatty acids in the blood (Souza et al., 1998). Thus, *Plin1* may play a key role in adipogenic and lipid metabolic processes. Moreover, *Plin1* expression may be specifically regulated by *PPAR-γ*, and activating *PPAR-γ* significantly increased *Plin1* expression, and *Plin1* may be a downstream target of *PPAR-γ*(Arimura et al., 2004). Our data exhibited that *Plin1* was significantly raised after BPBP treatment. Furthermore, we observed that *Adipoq* expression was also significantly upregulated after BPBP treatment. Adiponectin, a protein mainly secreted by adipocytes, is encoded by the *Adipoq* gene (Achari and Jain, 2017). A previous study showed that *Adipoq* overexpression allowed 3T3L-1 cells to differentiate into adipocytes more rapidly while exhibiting robust expression of *PPAR-γ* genes (Fu et al., 2005). Another study pointed out that the activation of *PPAR-γ* could up regulate the level of adiponectin (Nawrocki et al., 2006). This further supports that BPBP may promote cellular fat accumulation by affecting *PPAR-γ* signaling pathway.

Liver X receptorα (LXR-α), a transcription factor belonging to the nuclear receptor family, is encoded by *Nr1h3* (Laurencikiene and Rydén, 2012). Previous studies have shown that activating LXR-α during adipogenesis increases fat accumulation in 3T3L1 cells. The adipogenic effect of LXR-α is thought to be dependent on *PPAR-γ*, and activating *PPAR-γ* increased LXR-α expression in 3T3L-1 cells (Juvet et al., 2003; Seo et al., 2004). Our data exhibited that *Nr1h3* was significantly raised after BPBP treatment. This result also seems to support the notion that

BPBP may promote cell adipogenesis by activating *PPAR-γ*.

In a word, we explored the effect of BPBP on adipogenesis in 3T3-L1 cells using transcriptomic analysis. The results showed that BPBP may promote adipogenesis and fat accumulation in 3T3-L1 cells by activating the *PPAR-γ* signaling pathway. The *PPAR-γ* signal pathway diagram was drawn and shown in Fig. S1. BPBP may stimulated excessive expression of *PPAR-γ*, leading to upregulation of downstream genes such as *Plin1* and *Adipoq*, thereby promoting adipocyte differentiation and causing fat accumulation. This is the first report of BPBP promoting adipocyte differentiation and merit further investigations on the mechanisms of actions using other approaches including metabolomics or lipidomics underlying the disruption of lipid metabolism (Sun et al., 2022).

However, our present results are limited to a cellular model, and further *in vivo* studies combined with validation at the protein level are still needed in the future to fully understand the relationship of BPBP on adipogenesis and fat accumulation. In addition, the roles of other differentially expressed genes as well as pathways also remain to be further determined. Nevertheless, our work provides evidence for the potential obesogenic effect of BPBP as an industrial alternative for BPA, and may call for further research on the safety of the chemical in food products.

CRediT authorship contribution statement

Ge Wang: Conceptualization, Methodology, Investigation, Writing – original draft. **Yichao Huang:** Conceptualization, Writing – review & editing. **Yanpeng Gao:** Conceptualization, Writing – review & editing. **Ge Chen:** Data curation, Investigation, Software. **Leqi Cui:** Writing – review & editing. **Ye Peng:** Methodology, Validation, Writing – review & editing. **Quancai Sun:** Project administration, Funding acquisition, Methodology, Validation, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100534.

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