Integrated lipidomics and RNA sequencing analysis reveal novel changes during 3T3-L1 cell adipogenesis

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Abstract: After adipogenic differentiation, key regulators of adipogenesis are stimulated and cells begin to accumulate lipids. To identify specific changes of lipid composition and gene expression patterns during 3T3-L1 cell adipogenesis, we carried out lipidomics and RNA sequencing analysis of undifferentiated and differentiated 3T3-L1 cells. The analysis revealed significant changes in lipid content and gene expression patterns during adipogenesis. *Slc2a4* was up-regulated, which may be to enhance glucose transport; *Gpat3*, *Agpat2*, *Lipin1* and *Dgat* were also up-regulated, potentially to enrich intracellular triacylglycerol (TG). Increased expression of *Pnpla2*, *Lipe*, *Acsl1* and *Lpl* genes likely increase intracellular free fatty acids, which can then be used for subsequent synthesis of other lipids, such as sphingomyelin (SM) and ceramide (Cer). Enriched intracellular diacylglycerol (DG) can also provide more raw materials for the synthesis of phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), ether-PE and ether-PC, whereas high expression of *Pla3* may enhance the formation of lysophophatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). Therefore, in the process of adipogenesis of 3T3-L1 cells, a series of genes will be activated, resulting in great changes in the contents of various lipid metabolite in the cells, especially TG, DG, SM, Cer, PI, PC, PE, etherPE, etherPC, LPC and LPE. It provides a theoretical basis for our understanding of pathophysiology of obesity.

Keywords: 3T3-L1; Lipidomics; RNA sequencing; Triacylglycerol; Diacylglycerol; Sphingomyelin; Ceramide; Phospholipid

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Introduction

Adipogenesis is a multi-step process that is regulated by complex signaling network, resulting in dramatic changes in cell morphology (Jakab et al. 2021). After adipogenesis, mature adipocytes are occupied by large lipid droplets (Wang et al. 2013). Mouse 3T3-L1 preadipocytes can be used as a model for studying adipogenesis; a medium containing insulin, dexamethasone (Dex) and methylisobutylxanthine (IBMX) can induce the preadipocytes to differentiate. Insulin, a stimulator for insulin-like growth factor 1 (IGF-1), is critically important for adipogenic differentiation (Haeusler et al. 2018). Dex, an anti-inflammatory 9-fluoro-glucocorticoid, can inhibit proliferation of adipocytes. It also enhances the activity of several transcription factors required for differentiation, including members of the C/EBPs family, promoting terminal differentiation of adipocytes (Shugart & Umek 1997; Tomlinson et al. 2010). IBMX, a phosphodiesterase inhibitor, acts as a cAMP inducer and activates protein kinase A (PKA) to promote preadipocyte differentiation into adipocytes (Farmer 2006).

Several days after induction in vitro, fibroblast-like cells develop into round adipocyte like cells and begin to accumulate lipid droplets (LDs). Final differentiation occurs after seven to ten days of induction (Gabrielli et al. 2018). LDs consist of a lipid ester nucleus wrapped by a phospholipid monolayer. They are most conspicuous in white adipocytes, which have a large single-compartment lipid storage function. (Fujimoto & Parton 2011). Due to the prevalence of obesity, type 2 diabetes, and metabolic syndrome worldwide, regulation of lipid storage and utilization has become the focus of many studies. It is crucial to understand the changes and mechanisms of adipogenesis to develop therapeutic strategies for these diseases.

Large numbers of lipid droplets accumulate in mature adipocytes. However, the species of enriched lipids, and how the lipid profile changes over the course of adipogenesis, are still unknown. Therefore, in the current study, we combined lipidomics and RNA sequencing to analyze differences in lipid content and lipid-associated genes between undifferentiated and differentiated 3T3-L1 cells.

Materials and Methods

Cell culture and 3T3-L1 cell differentiation

3T3-L1: The 3T3-L1 cells were obtained from Prof. Shulin Yang (Institute of Animal sciences, Chinese Academy of Agricultural Sciences). The maintaining medium supplemented with DMEM (Gibico), 10% FBS (Gibico) and 1% penicillin-streptomycin (Gibico) was used to maintain 3T3-L1 cells. 1×106 cells/well were cultured in 6-well cell culture plates. These cells were grown until confluence (day 0), then differentiation was induced by adding 1 mM insulin (Sigma I5523), 0.25 mM dexamethasone (Sigma D175) and 0.5 mM 3-isobutyl-1- methylxanthine (Sigma 15879) in maintaining medium. After 4 days, cells were cultured in maintaining medium with 1 mM insulin. On day 10, the fully differentiated adipocytes were used for oil red staining. The cells of day 0 and day 10 were collected and stored in -80°C for subsequent experiments.

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Nile Red O Staining

Cells were treated with 4% PFA for 10 min. Afterwards, cells were washed with PBS and incubated with Nile Red O (sigma) for 30 min. After staining, images were acquired using a LEICA DMi8 microscope (Leica DMi8; Leica Microsystems, Inc.).

Lipid extraction and Lipidomics study

Collected cells were thawed on ice, and lipids were extracted with isopropanol (IPA). Samples were added 120 μL precooled IPA, then vortexed for 1 min, and incubated for 10 min at room temperature. The extraction mixture was then stored at -20°C overnight. After centrifugation at 4,000 g for 20 min, the supernatants (one per specimen) were diluted to 1:10 with IPA/acetonitrile (ACN)/H_2O (2:1:1, v:v:v) and stored at -80°C before analysis. 10 μL of each extraction mixture was removed to prepare the pooled QC samples.

All lipid samples were analyzed by liquid chromatography (LC)- mass spectrometry (MS) using ultra-high performance liquid chromatography (UPLC) system (SCIEX, UK) and high-resolution tandem mass spectrometer TripleTOF5600plus (SCIEX, UK). The acquired LC–MS raw data were analyzed by XCMS software (SCIEX, Warrington, UK), and the retention time (RT) and M/Z data were used to identify each ion. The online KEGG, HMDB and in-house databases were used to perform level-one and level-two identification and annotation. MS2 indicates the metabolites that not only match with level-one fragment ion, but could also match with level-two fragment ion in the database. Lipid metabolites with VIP (Variable Important for the Projection) ≥1 and fold change >2 or <−2 were considered statistically significantly different (Li et al. 2018).

2.4 mRNA library construction and sequencing

Trizol reagent (Invitrogen) was used to exacted total RNA from cells in accordance with the manufacturer's procedure. Then we analyzed the RNA quantity and purity. Poly-T oligo-attached magnetic beads was used to purify Poly(A) RNA from total RNA with two rounds of purification. After purification, the mRNA was divided into small pieces at high temperature using divalent cations. Then, according to the procedures of the mRNA Seq sample preparation kit, the cut RNA fragments were reversely transcribed to generate the final cDNA library (Illumina, San Diego, USA). FastQC (version 0.11.2) was used for evaluating the quality of sequenced data. HISAT2 (version 2.0.4) was used to get clean data comparing to the genome (Mus musculus, Ensembl v101)(Kim et al. 2015). StringTie (version 1.3.4) and Gffcompare (version 0.9.8) were used to assemble and quantify the transcripts, respectively (Pertea et al. 2015; Pertea et al. 2016). Differential gene expression between two groups (three biological replicates per condition) was performed using the DESeq2 R package available from Bioconductor (Love et al. 2014). Genes with an adjusted p-value < 0.05 and fold change >2 or < −2 found by DESeq were assigned as differentially expressed. The statistical power, calculated using RNASeqPower

(https://doi.org/doi:10.18129/B9.bioc.RNASeqPower) is 0.9829191. RNA sequencing data can be accessed on the SRA database, accession numbers PRJNA795061

2.5 Quantitative real time PCR (qRT-PCR)

qRT-qPCR was performed using TaKaRa SYBR Premix EX Taq (TaKaRa RR420A, JAPAN) on a QuantStudio5 Real-Time PCR System (Applied Biosystems, USA). All of the experiments contained three biological replicates, and each sample was quantified in duplicate. SPSS software and Excel were used to analyze the data. Primer sequences are shown in Table S1.

2.6 Correlational analysis

Three sets of transcriptome data and three sets of lipidomics data were used for correlation analysis. Correlation analysis was performed using the OmicStudio tools at https://www.omicstudio.cn/tool.

3. Results

3.1 3T3-L1 adipogenesis

Oil Red O staining showed that accumulation of lipid droplets within the cells increased during induction of differentiation (Figure 1 A-F). In order to identify the changes between undifferentiated and differentiated 3T3-L1 cells, we collected samples at day 0 and day 10 for subsequent testing.

3.2. LC-MS-based untargeted lipidomics

We analyzed a total of 12 samples from 3T3-L1 preadipocyte and mature adipocyte samples (n=6 per group) by LC-MS-based untargeted lipidomics to reveal difference in lipid composition. A total of 5335 features were detected in positive ion mode, of which 3187 could be annotated, with 850 matches to in-house database. A total of 1554 features were detected in negative anion mode, 873 of which could be annotated, with 378 matches to in-house database. A total of 1228 MS2 metabolites were identified from positive and negative ion modes (Table S2). Among all of the detected features, there were more up-regulated metabolites than down-regulated metabolites in mature fat cells (Figure 2 A).

Further lipid profiles were compared using principal component analysis (PCA) (Figure 2 B). In unsupervised mode, the samples clustered together by cell type. Partial least squares Discriminant Analysis (PLS-DA) was used to identify the altered metabolites, and we found significant differences between the D0 and D10 groups (Figure 2 C, D). These results demonstrated significant lipid metabolite changes during differentiation of 3T3-L1 cells.

The *q*-values obtained by Benjamini-Hochberg (BH) correction using univariate fold-change analysis, the *t.test*, and the VIP value obtained by PLS-DA were analyzed by multivariate statistical analysis and used to screen for differentially expressed lipid metabolites. Based on VIP values and relative abundance, we identified 454

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differentially expressed MS2 metabolites between D10 and D0, among which 301 metabolites were up-regulated and 153 metabolites were down-regulated (Figure 3A). These differentially expressed metabolites included 214 glycerophospholipids (GPs), 152 glycerolipids (GLs), 73 sphingolipids (SPs), 10 fatty acyls (FAs), three sterol lipids (ST) and two prenol lipids (PLs) (Figure 3B, Table S3). Among the 214 differentially expressed GPs, there were 50 PCs (26 decreased, 24 increased), 36 LPCs (16 decreased, 20 increased), 29 EtherPEs (17 decreased, 12 increased), 23 PE (4 decreased, 19 increased), 17 PI (1 decreased,16 increased), 11 EtherPC (8 decreased, 3 increased), 11 LNAPEs (1 decreased,10 increased) and nine LPEs (2 decreased, 7 increased). Among the 152 differentially abundant GLs, there were 66 TGs (2 decreased, 64 increased), 27 EtherTGs (4 decreased, 23 increased), 25 DGs (all increased), and 23 EtherMGDGs (18 decreased, 5 increased). The 73 differentially expressed SPs included 28 SMs (16 decreased; 12 increased), 12 Cer_NS (3 decreased, 9 increased), nine HexCer_NS (4 decreased, 5 increased) and eight SHexCer (5 decreased, 3 increased).

The top 10 down-regulated lipids were PS 18:0_22:6, LPC 30:0-SN1, CAR 18:1, CAR 16:0, PE O-18:2_22:5, LPC 36:2-SN1, PC O-18:1_18:1, LDGCC 40:6, PE 38:4, and SMGDG O-11:0_28:6. The top 10 up-regulated lipids were all TGs: TG 16:1_16:1_17:1, TG 15:0_16:0_16:1, TG 14:1_16:1_17:1, TG 10:0_16:0_16:1, TG 16:0_16:1_16:2, TG 14:1_16:1_16:1, TG 14:0_16:1_16:1, TG 14:0_15:0_16:1, TG 8:0_16:0_16:1, and TG 14:0_14:1_16:1 (Table 1 and Table S3).

The differentially expressed lipids were analyzed for biochemical pathway enrichment using the Kyoto Encyclopedia of Genes and Genomes database (Figure 4). We found that differentially regulated lipid molecules in the mature adipocytes were broadly related to metabolism (eg., ether lipid metabolism and sphingolipid metabolism) and organismal systems (e.g., adipocytokine signaling pathway and regulation of lipolysis in adipocytes).

3.3 Differential gene expression between undifferentiated and differentiated 3T3-L1 cells

Cell samples at D0 and D10 were analyzed via RNA-sequencing (RNA-seq). The main characteristics of the libraries are shown in table S4. They contained 49,519,629 raw reads on average. After removing adaptors and low-quality/ambiguous sequences, an average of 47,209,124 valid clean reads remained. Among all samples, 96.45% of the valid reads mapped to the mouse genome database, including 72.76% unique mapped reads and 23.69% multi-mapped reads (Table S5). The gene expression profiles of D0 and D10samples were analyzed with PCA. This analysis revealed significant differences in gene expression patterns between D10 and D0 (Figure 5A). Volcano plots were used to visualize the distribution of differentially expressed genes (DEGs) between D10 and D0 cells. There were 6193 DEGs between D10 and D0, including 1878 up- and 4315 down-regulated genes (Figure 5B). Heatmap comparison represents some gene expression changes (Figure 5 C).

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We next validated the RNA-seq data with qRT-PCR for randomly selected genes. The relative expression levels of acyl-Coenzyme A dehydrogenase medium chain (*Acadm*), acyl-CoA synthetase long-chain family member 1 (*Acsl1*), angiopoietin-like 4 (*Angpt14*), fatty acid binding protein 5 (*Fabp5*), lipase hormone sensitive (*Lipe*), stearoyl-Coenzyme A desaturase 1 (*Scd1*), patatin-like phospholipase domain containing 2 (*Pnpla2*), stearoyl-Coenzyme A desaturase (*Scd1*) and lipoprotein lipase (*Lpl*) were significantly increased in differentiated 3T3-L1 cells (D10, $p < 0.05$, Figure 6). The expression trends for all genes validated by qRT-PCR were consistent with the results from the RNA-seq analysis, demonstrating the high quality of the sequencing data.

The 6193 DEGs identified with RNA-seq were analyzed for functional enrichment using the Gene Ontology (GO) database. There were significantly enriched biological process (e.g. lipid metabolic process, fatty acid biosynthetic and lipid transport), cellular components, and molecular functions (Figure S1). Enrichment analysis was also conducted using the KEGG database (Figure 7). The integrated DEGs of undifferentiated and differentiated 3T3-L1 cells were mainly enriched in thermogenesis, the PPAR signaling pathway, and regulation of lipolysis in adipocytes.

3.4 Joint analysis of RNA-seq and lipid metabolome results

We compared the enriched KEGG pathway between differentially expressed genes and differentially expressed lipid metabolites. There were seven pathways enriched in both datasets (p<0.05), namely adipocytokine signaling pathway, AGE-RAGE signaling pathway in diabetic complications, glycerolipid metabolism, insulin resistance, oxidative phosphorylation, regulation of lipolysis in adipocytes, and retrograde endocannabinoid signaling (Figure 8 and Table S5). Genes with a fold change > 5 and FPKM > 1 (Table 2) were strongly correlated with metabolites in these pathways (Figure S2 and Table S6). All of the differentially expressed genes were screened and a protein interaction network was constructed using String. The key hub genes were identified with Cytohubba in Cytoscape. The top 10 hub genes were *Pnpla2*, diacylglycerol O-acyltransferase 1 (*Dgat1*), diacylglycerol O-acyltransferase 2 (*Dgat2*), fatty acid binding protein 4 (*Fabp4*), adiponectin C1Q and collagen domain containing (*Adipoq*), *Lipe*, *Acsl1*, solute carrier family 2, member 4 (*Slc2a4*), *Lpin1* and *Lpl* (Figure 9).

3.5 Expression patterns of hub genes and key genes that regulate lipid metabolism during adipogenesis

Expression patterns of the top 10 hub genes were analyzed in cell samples collected at D0, D2, D4, D6, D8, and D10 cell during adipogenesis. In addition, the expression of the key adipogenesis genes CCAAT enhancer binding protein alpha (*Cebp/α*) and peroxisome proliferator activated receptor gamma (*Pparγ*) were also quantified; both were significantly activated during differentiation (Figure S3). *Pnpla2* (Figure 10A), *Dgat1* (Figure 10B), *Fabp4* (Figure 10D), and *Lipe* (Figure 10F) were significantly upregulated during differentiation, with the highest expression at day 4. *Dgat2* was

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highly expressed at D2 and D4 during differentiation, but the expression level was lower than in undifferentiated cells at D10 (Figure 10C). *Adipoq* (Figure 10E), *Acsl1*(Figure 10G), *Lpin1* (Figure 10H), *Lpl* (Figure 10I), and *Plin1* (Figure 10J) were significantly upregulated during differentiation, with the highest expression at day 8.

4. Discussion

In this study, we integrated lipidomics and RNA sequencing to reveal significant changes in lipid content and gene expression profiles between undifferentiated and mature 3T3-L1 cells during adipogenesis. Previous research examined the lipid change between undifferentiated and differentiated 3T3-L1 cells. However, they only analyzed the changes of SM, PC, TG, PI, PE and FA (Popkova et al. 2020). Here, we found that in addition to the changes above mentioned, there were many other lipids which showed significant changes after differentiation, such as LPC, EtherPE and DG. Furthermore, many studies have conducted the transcriptome sequencing during adipogenesis, and a large number of DEGs related to the initiation of adipogenesis have been identified (Mikkelsen et al. 2010; Duteil et al. 2014; Al Adhami et al. 2015; Siersbæk et al. 2017; Romero et al. 2018). But the results are quite variable, which could be due to different sample batches, testing platforms and data processing methods. So, we combined lipidomics and transcriptomic studies, that can provide a better understanding of the molecular mechanism of adipogenesis.

As expected, compared with undifferentiated 3T3-L1 cells, there were more TGs and DGs fraction, especially TGs, in differentiated 3T3-L1 cells. This is consistent with a previous study that found that adipocytes have a higher level of TGs than cells in the preadipocyte state (Popkova *et al.* 2020). Upon insulin stimulation, Slc2a4 moves to the cell surface and transports glucose from the extracellular milieu into the cell (Watson et al. 2004). GPAT3 has catalytic activity for a variety of saturated and unsaturated long-chain fatty acyl-CoAs, such as oleoyl-CoA, linoleoyl-CoA, and palmitoyl-CoA (Cao et al. 2006). During adipogenesis GPAT activity is increased by 30- to 100-fold (Coleman et al. 1978). AGPAT2 catalyzes the second step of TG synthesis (the glycerol phosphate pathway, the main synthesis pathway of triacylglycerol), which is highly expressed in adipose tissues (Gale et al. 2006). Lipins, PAP enzymes, can catalyze the dephosphorylation of phosphatidate to diacylglycerol in TG biosynthesis (Péterfy et al. 2001; Csaki et al. 2013). Lipin-1 plays a key role in adipose tissue PAP activity (Kok et al. 2012). In differentiating preadipocytes, Lipin-1 is required for normal expression of key adipogenesis regulating genes, including PPARγ and C/EBP α, and for the synthesis of TG (Zhang et al. 2008). DGAT catalyzes DG to form, it has DGAT1 and DGAT2 two isoforms (Shi & Cheng 2009). Functional DGAT is required for LDs in adipocytes (Harris et al. 2011). ACSL1 has important role in activating fatty acid synthesis of triglycerides (Li et al. 2009). A prior study showed

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that when Acsl1 was overexpressed in mouse hearts, triglyceride levels in cardiomyocytes increased by 12-fold (Chiu et al. 2001).The high expression of *Slc2a4* indicated that cellular glucose transport capacity was enhanced during adipogenesis. We here found upregulation of *Gpat3*, *Agpat2*, *Lipin1*, *Dgat1*, *Dgat2* and *Acsl1* during adipocyte differentiation, suggesting that *Gpat3*, *Agpat2*, *Lipin1*, *Dgat1*, *Dgat2*, and *Acsl1* transcriptionally control TAG synthesis during the adipogenesis of 3T3-L1 cells (Figure 11).

We identified 73 sphingolipids (SPs) with significantly different levels between undifferentiated and differentiated adipose cells. Among these SPs, the most drastic changes were in levels of SM and Cer_NS. De novo ceramides synthesis begins with the condensation of palmitate and serine to form 3-keto-dihydrosphingosine. 3-keto-dihydrosphingol is then reduced to dihydrosphingol, which is subsequently acylated by the enzyme (dihydroceramide) synthetase to produce dihydroceramide. The final reaction of ceramide formation is catalyzed by dihydroceramide desaturase. Ceramide can be further metabolized to other sphingolipids, such as sphingomyelin and Cer_NS (Ramstedt & Slotte 2002). Pnpla2 encodes adipose triglyceride lipase (ATGL) and Lipe encodes HSL in adipose tissue. ATGL and HSL are the major enzymes that promote the decomposition of TG in mouse white adipose tissue. ATGL performs the first step in TG catabolism generating diacylglycerol and fatty acids. Diacylglycerol is subsequently degraded by HSL and monoglyceride lipase (MGL) into glycerol and fatty acids (Zimmermann et al. 2004; Gao & Simon 2007). LPL encodes lipoprotein lipase which catalyzes the hydrolysis of triglycerides (Pingitore et al. 2016). Fabp4 encodes the fatty acid binding protein that binds long chain fatty acids and retinoic acid, and delivers them to their cognate receptors in the nucleus (Prentice et al. 2019). The high expression of *Pnpla2*, *Lipe*, and *Lpl* suggests that the level of intracellular free fatty acids will increase and can be used for synthesis of other lipids. Fabp4 can then transport these metabolites to specific sites for further synthesis of other lipids, such as ceramide and sphingomyelin (Figure 11).

We also found that there were 214 GPs with significantly different levels between undifferentiated and differentiated cells. The GPs with the most differences were PI, PC, PE, LPE, LPC, ether-PE and ether-PC. De novo formation of PE and PC in eukaryotes occurs through several pathways. PE can be synthesized through the cytidine diphosphate (CDP)-ethanolamine branch of the Kennedy pathway, whereas PC can be synthesized through the CDP-choline branch of the Kennedy pathway (Kennedy & Weiss 1956) or methylation of PE (Bremer et al. 1960). Biosynthesis of PI is catalyzed by phosphatidylinositol synthase (PIS), which produces phosphatidylinositol and cytidine-monophosphate from the substrate molecules inositol and CDP-diacylglycerol (Bankaitis & Grabon 2011). Phospholipase A2 can convert PC and PE

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into LPC and LPE, respectively. (Makide et al. 2009; Liu et al. 2017). Plaat3 has phospholipase A1 and A2 activity (Mardian et al. 2015), and can therefor catalyze the release of fatty acids from glycerophospholipids (in the sn-1 or sn-2 position) to form lysophospholipid (Pang et al. 2012). Our lipidomics analysis showed that production of many DGs (eg. DG 16:0_17:1 and DG 15:0_16:0) was increased during adipogenesis. The increased in DG abundance could provide more sources for the synthesis of PI, PC, PE, ether-PE, and ether-PC. High expression of *Plaat3* (Table 2) may be related to enrichment of LPC and LPE.

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Conclusions

In conclusion, in the process of adipogenesis in 3T3-L1 cells, a series of genes, such as *Slc2a4*, *Gpat3*, *Agpat2*, *Lipin1*, *Dgat1*, *Dgat2* and *Acsl1* were activated, resulting in large amounts of TG accumulate. In addition to TG synthesis regulated genes, some other lipid-regulated genes, such as *Pnpla2*, *Lipe*, and *Lpl* were also up-regulated, leading to changes in the content of other lipids such as SM, Cer, PI, PC, PE, etherPE, etherPC, PLC and PLE. It provides a reference for understanding the mechanism of human obesity development, and a basis for finding effective ways to prevent obesity.

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Author Contributions: YP and ZF designed and managed the project. YP, YS and CL analyzed the data and performed all cell works and collected biological samples. YP wrote the manuscript. ZF, BW, and HL revised the paper. All authors approved the final version of the manuscript.

Funding: This work was supported by the National Science Foundation for Young Scientists of China (31702088), the Guangdong Provincial Key Laboratory of Animal Molecular Design and Precise Breeding (2019B030301010), the Key Laboratory of Animal Molecular Design and Precise Breeding of Guangdong Higher Education Institutes (2019KSYS011), and Foshan University Initiative Scientific Research Program.

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Figure legend

Figure 1 Oil Red O staining of 3T3-L1 cells during adipogenesis

Oil Red O staining of 3T3-L1 cells at D0 (A), D2 (B), D4 (C), D6 (D), D8 (E), and D10 (F) after duction. The red dots inside the cells are lipid droplets.

Figure 2 Volcano plot, PCA and PLS-DA of the detected compounds in the two groups

(A) Volcano plot of lipid metabolites.

(B) PCA scatter plot of differentially expressed lipid metabolites.

(C) PLS-DA score plots of D0 and D10 cells based on the extracted spectral data.

(D) Permutation plot of PLS-DA based on the extracted spectral data.

Figure 3 Differentially expressed lipid metabolites in two groups

(A) Among 454 identified differentially expressed metabolites, 301 were upregulated and 153 were downregulated.

(B) The differentially expressed lipid metabolites included 214 glycerophospholipids (GPs), 152 glycerolipids (GLs), 73 sphingolipids (SPs), 10 fatty acyls (FAs), three sterol lipids (STs) and two prenol lipids (PLs).

Figure 4 Lipid metabolic pathway analysis of the identified differentially expressed lipid species

Figure 5 RNA-seq analysis of undifferentiated and differentiated 3T3-L1 cells

A. PCA results of the two groups.

B. Volcano plot of DEGs in undifferentiated and differentiated 3T3-L1 cells. Down-regulated genes are represented by blue dots and up-regulated genes are represented by red dots.

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C. Heatmap comparison represents gene expression changes.

Figure 6 Gene expression levels from RNA-seq analysis (FPKM) and qRT-PCR (Relative Expression)

qRT-PCR was used to analyze expression levels of *Acadm*, *Acsl1*, *Angptl4*, *Fabp5*, *Lipe*, *Pnpla2*, *Scd1*, *Npy1r*, *Pik3cd*, and *Lpl*. The *18s*, *B2m*, and *β-actin* genes were used as internal references for standardization. Bar graph (Blue) showing results from qRT-PCR (left ordinate). The line chart represents the results from RNA-seq analysis (right ordinate in red).

Figure 7 Bubble diagram of KEGG enrichment result

Bubble color corresponds to the *p* value for statistical significance of KEGG pathway enrichment. Bubble size is proportional to the number of genes annotated in a particular pathway.

Figure 8 Histogram showing *p* values for statistically significant KEGG pathway enrichment overlapping between the differentially expressed gene and differentially expressed metabolite datasets

Figure 9 Interaction network of the top 10 hub genes

Figure 10 Relative expression of top 10 hub gene

qRT-PCR was used to analyze expression of the top 10 hub genes. *18s*, *B2m*, and *β-actin* served as the internal reference genes.

Figure 11 Regulation pathways of genes and lipid metabolites during 3T3-L1 adipogenesis

The yellow circles represent the differentially expressed lipid metabolites, and red text represent differentially expressed genes.

Table 1 The top 10 down-regulated and up-regulated lipids in D10 versus D0 groups

Table 2 Differentially expressed genes (fold change > 5 and FPKM > 1) in the seven pathways enriched in both datasets.

Figure S1 Gene ontology (GO) term enrichment analysis,

Figure S2 Heat map of correlation analysis between differentially expressed genes and lipid metabolites,

Figure S3 The expression of *Cebp/α* and *Pparγ* in adipogenesis detected by RT-qPCR,

Table S1: The sequence of primers.

Table S2 LC-MS-based untargeted lipidomics results

Table S3 Significant differentially expressed lipid metabolites in D10 versus D0 groups

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下へ移動 [1]: Table 1 LC-MS-based untargeted lipidomics results

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Table S4 Summary of sequencing analysis in RNA-seq,

Table S5 The data for the sequencing reads that mapped to the reference genome in RNA-seq,

Table S6 The seven pathways enriched in both datasets,

Table S7 The correlation between DEG and differentially expressed lipid metabolites generated using Pearson's correlation coefficient.

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