Identification of Differentially Expressed Genes for Lipid Metabolism in Dedifferentiated Preadipocytes from Different Tissues of Chicken

zheng ma
Foshan University
Na Luo
Chinese Academy of Agricultural Sciences
Lu Liu
Chinese Academy of Agricultural Sciences
Huanxian Cui
Chinese Academy of Agricultural Sciences
Jing Li
Foshan University
Hai Xiang
Foshan University
Huimin Kang
Foshan University
Hua Li
Foshan University
Guiping Zhao (zhaoguiping@caas.cn)
Foshan University

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Abstract

Background: The body distribution with high intramuscular fat and low abdominal fat is ideal goal for broiler breeding. Preadipocytes with different origins have differences in metabolism and gene expression. This transcriptome analysis of intramuscular preadipocytes (DIMPs) and adipose tissue-derived preadipocytes (DAFPs) is aim to explore the characteristics in lipid deposition of different chicken preadipocytes by dedifferentiation in vitro.

Results: Compared to DIMFPs, the lipid content was increased (P <0.05) in DAFPs after two days with 100% confluence. Moreover, 66 DEGs of lipid metabolism were screened, which are involved in the adipocyte differentiation, fatty acid transport and fatty acid synthesis, lipid stabilization, and lipolysis. Among them, the representative *CEBPA*, *DGKH*, *DGKQ*, *DGKD*, *FADS1L1*, *SCD*, *SCD5*, and *PPARG* were down-regulated, but *CIDEC*, *ELOVL1*, *ELOVL6*, *FABP3*, *FABP4*, *FADS6*, *LPL*, *MOGAT1*, *PLIN3*, *PLIN4*, *RBP7*, and *RXRG* genes were up-regulated (P < 0.05 or P < 0.01) in the DAFPs, showing the same pattern with the lipid content. Based on the known DEGs, the well-known pathways affecting lipid metabolism (MAPK-, TGF beta-, Calcium-, PPAR signaling pathway) were enriched, which may also contribute to the regulation of lipid deposition.

Conclusions: Our data suggest that the difference of lipid deposition between DIMPs and DAFPs of chicken in vitro. The lipid content was significantly increased in DAFPs by the up-regulation of genes on cellular uptake of fatty acids through medication of MAPK-, TGF beta-, Calcium-, and PPAR signaling pathways. These findings provide new insights into the regulation of tissue-specific fat deposition and optimizing body fat distribution in broilers.

Background

Fat has unique distribution characteristics and different economic value in various tissues of animals. In broilers, high-intensity artificial breeding has effectively increased meat yield, but also increased abdominal fat content and reduced intramuscular fat deposition [1]. Excessive abdominal fat deposition has negative impacts on feed efficiency and carcass yield [2, 3]. Decreased abdominal fat deposition is beneficial to reduce produces waste and improve consumer acceptance. In contrast, intramuscular fat is economically desirable in broiler production. Appropriately increased IMF content can improve meat quality, including color, tenderness, flavor and juiciness [4–7]. Lowering abdominal fat and increasing intramuscular fat can effectively increase the economic value of broilers.

Previous studies have showed that adipocytes with different origins exhibited differential differentiation capabilities [8]. Compare with subcutaneous preadipocytes, the cell size and lipid droplets in intramuscular adipocytes are smaller [9, 10], and the gene expression and enzyme activation related to lipid metabolisom were lower in intramuscular adipocytes [11, 12]. Similarly, abdominal fat-derived preadipocytes exhibited a higher adipogenic differentiation ability than intramuscular fat-derived

preadipocytes in chickens [13, 14]. However, it is still unknown whether the difference on lipogenesis ability of preadipocytes from different tissues will disappear after cultivating in vitro.

In this study, we explored the characteristics in lipogenesis of chicken preadipocytes with different origins after cultivating in vitro, including the dedifferentiated intramuscular preadipocytes (DIMFPs) and the dedifferentiated abdominal preadipocytes (DAFPs). These results will help to understanding tissue-specific lipid deposition and optimizing body fat distribution in broilers.

Results

Identification of DEGs related to lipid metabolism in two-type preadipocytes

Each three cell repetitions of DIMFP and DAFP group were used for RNA extraction. Using gene expression profiling and comparing the DAFP group with the DIMFP group (DIMFP vs DAFP) (Fig. 1a), a total of 3486 known DEGs were screened, including 2579 down-regulated and 907 up-regulated (Additional file 1:Table S1). Based on these 3486 DEGs, cluster analysis was performed, and the results showed that cell samples of the same groups were clustered together, respectively (Fig. 1b).

GO enrichment analysis were performed based on 3486 known DEGs. The enriched 56 GO terms were screened and mainly included the following processes: cell adhesion, tight adhesion, cell differentiation, extracellular matrix, DNA binding, cacium ion binding, et al. (Additional file 2:Table S2), each the top 10 terms of biological process (BP), cellular component (CC) and molecular function (MF) were shown in Fig. 2. As expected, GO enrichment analysis indicated that 66 DEGs related to lipid metabolism, and some representative DEGs were screened (Additional file 3:Table S3).

Changes of DEGs related to lipid metabolism in two-type preadipocytes

Without differentiation induction, the DIMFP and DAFP cells after 2 days with 100% confluence were simultaneously collected for the detection of lipid content, and data were depicted in Fig. 3a. The result showed that lipid content in DAF cells was significantly ($P < 0.05$) higher than that in DIMF cells.

For the DEGs related to lipid metabolism, they mainly involved in the adipocyte differentiation (such as *CEBPA*, *PPARG*, *RBP7*, and *RXRG*), fatty acid transport and fatty acid synthesis (such as *ELOVL1*, *ELOVL6*, *FABP3*, *FABP4*, *FADS6*, *FADS1L1*, *SCD*, and *SCD5*), lipid stabilization (such as *CIDEC*, *PLIN3*, *PLIN4*, and *MOGAT1*), and lipolysis (such as *DGKD*, *DGKH*, *DGKQ*, and *LPL*). The 20 representative DEGs were selected to validate the gene expression profiling results by qRT-PCR, and the correlation of gene expression profiling and qRT-PCR were analyzed by Spearman rank correlation to confirm the accuracy of the data. The result showed that the fold change of gene expression between the two methods was significantly correlated (Fig. 3b) ($r = 0.9666$, $P < 0.01$). Among them,the expression levels of genes (*CEBPA*,

DGKH, *DGKQ*, *DGKD*, *FADS1L1*, *SCD*, *SCD5*, and *PPARG*) had been significantly ($P < 0.05$ or $P < 0.01$) downregulated in DAFPs compared to DIMFPs (Fig. 3c). However, It was found that the expression changes of *CIDEC*, *ELOVL1*, *ELOVL6*, *FABP3*, *FABP4*, *FADS6*, *LPL*, *MOGAT1*, *PLIN3*, *PLIN4*, *RBP7*, and *RXRG* genes had been significantly (all $P < 0.01$) up-regulated in DAFPs compared to DIMFPs (Fig. 3d), showing the same pattern with the lipid content.

Pathways involved in the lipid metabolism in two-type preadipocytes

Also, 47 pathways were found to be significantly enriched (Corrected *P*-value < 0.05) (Additional file 4:Table S4), including some well-known pathways affecting lipid metabolism (MAPK-, TGF beta-, Wnt-, Calcium-, PPAR signaling pathway) and the other pathways related to cell communications (Focal adhesion, Cytokine-cytokine receptor interaction, ECM-receptor interaction, Tight junction, Regulation of actin cytoskeleton, Cell adhesion molecules, Adherens junction). The top 15 pathways were shown in Fig. 4.

Among them, Some representative genes related to lipid metabolism were enriched in the PPAR signaling pathway (Additional file 6:Figure S1), including to *FABP3*, *FABP4*, *LPL, PPARG*, *RXRG*, *SCD*, and *SCD5 et al.* Similarly, there also had a large number genes were enriched in MAPK-, Calcium-, and TGF beta signaling pathway, and the evidence pointed to these three pathways could mediate the biology function of cell differentiation or metabolism in this study (Additional file 7:Figure S2, Additional file 8:Figure S3, and Additional file 9:Fig. 4). However, it was found that the enriched Wnt signaling pathway, as the well-known pathways affecting lipid metabolism, did’t medicate the regulation on lipid metabolism.

Discussion

Fat has unique distribution characteristics and different economic value in various tissues of animals. In broilers, the intramuscular fat is economically desirable in production. Appropriately increased IMF content can improve meat quality, including tenderness, flavor and juiciness [4–6]. However, excessive abdominal fat deposition has negative impacts on feed efficiency and carcass yield [2, 3], and decreased abdominal fat deposition is beneficial to reduce produces waste and improve consumer acceptance. Lowering abdominal fat and increasing intramuscular fat can effectively increase the economic value of broilers. Therefore, how to change the constitution distribution is an important scientific problem for broilers.

Unlike a marbling distribution of IMF in domestic animals, the IMF of chicken cannot be obtained directly from anatomy. Moreover, muscle tissue of chicken has a variety of cell composition [15], the preadipocytes of IMF can not be separated by physical methods for the similar density with muscle cells. So, the high purity preadipocytes of IMF can only be obtained by the dedifferentiation of mature adipocytes in vitro as described previously [16]. In this study, the abdominal fat preadipocytes and intramural preadipocytes were respectively obtained from the mature adipocytes of the same chicken to compare their lipogenesis ability with the consistency of experimental conditions in vitro, expecting to

establish a theoretical foundation for the body fat distribution of chicken and provide ideas and development direction for chicken production. As known, the adipocytes in different tissues were regulated by the adjacent microenvironment to perform the corresponding physiological function [17, 18]. To eliminate the effect of factors in vivo and in vitro, the second-generation cells used. After the cells were overgrown for 2 days, the lipogenesis of adipocytes were detect, which was different from the usual practice of inducing adipocyte differentiation in vitro, avoiding the possibility that the inducers could conceal the lipogenesis of the cells themselves. The results showed that the lipogenesis of preadipocytes derived from abdominal adipocytes were significantly superior to those derived from muscle tissue, consistent with those results in vivo as previously reported [19, 20].

Genetically, the molecular basis on the difference in lipid deposition between the two cells was explored using RNA-sEq. Transcriptome results revealed a large number of differentially expressed genes involved in the biological processes, such as cell adhesion, tight adhesion, cell differentiation, extracellular matrix, DNA binding, cacium ion binding, et al. Also, the genes related to lipid metabolism were identified, including some representative genes related to lipid metabolism. These supported the differences in lipid deposition between the two types of cells. Further, the expression levels of some classical genes were verified by qRT-PCR. These representative lipid metabolism related genes mainly involved in intracellular fat decomposition (*DGKH*, *DGKQ*, and *DGKD*) [21, 22], extracellular fat decomposition (*LPL*) [23], fatty acid synthesis (*ELOVL1*, *ELOVL6*, *FADS1L1*, *FADS6*, *SCD*, and *SCD5*) [22–25], fatty acid transport (FABP3 and FABP4) [26, 27], fat maintenance (*CIDEC*, *MOGAT1*, *PLIN3*, and *PLIN4*) [28–31] and adipocyte differentiation (*PPARG*, *RBP7*, and RXRG) [32, 33]. The correlation analysis on data of RNA-seq and qRT-PCR showed that they had a strong positive correlation, which confirmed the accuracy of RNA-seq data. Among these representative genes, the *CEBPA*, *DGKH*, *DGKQ*, *DGKD*, *FADS1L1*, *SCD*, *SCD5*, and *PPARG* expressions were down-regulated in DAFPs compared to DIMFPs, but the *CIDEC*, *ELOVL1*, *ELOVL6*, *FABP3*, *FABP4*, *FADS6*, *LPL*, *MOGAT1*, *PLIN3*, *PLIN4*, *RBP7*, and *RXRG* expressions were up-regulated in DAFPs compared to DIMFPs, showing the consistency with the lipid content. So it was considered that these gene had the important effects on regulating the lipid deposition in adipocyte of chicken.

Based on KEGG database, 47 enriched pathways were screened, including the well-known pathways affecting lipid metabolism (MAPK-, TGF beta-, Wnt-, Calcium-, PPAR signaling pathway). Among them, it was acknowledged that the PPAR signaling pathway plays a key role in mediating lipid metabolism [34, 35]. Here, the representative genes related to lipid metabolism (*FABP3*, *FABP4*, *LPL, PPARG*, *RXRG*, *SCD*, and *SCD5 et al.*) were enriched in the PPAR signaling pathway. In addition, it was reported that MAPK-, TGF beta-, Wnt-, and Calcium signaling pathways had the interaction with the PPAR pathway to regulate the lipid metabolism in the lipogenesis process [36–38], and there also had a large number genes were enriched in MAPK-, Calcium-, and TGF beta signaling pathways. According to the enrichment information of these three signaling pathways in this study, the evidence pointed to these three pathways could mediate the biology function of cell differentiation or metabolism. Then, it was deduced that the MAPK-, Calcium-, and TGF beta signaling pathways also involved the regulation of lipogenesis between DAFPs and DIMFPs.

Conclusions

In brief, our data suggest that the difference of lipid deposition in DIMPs and DAFPs of chicken obtained by dedifferentiation in vitro. The lipid content was significantly increased in DAFPs by the up-regulation of genes on cellular uptake of fatty acids, which promotes lipid deposition through medication of MAPK-, TGF beta-, Calcium-, and PPAR signaling pathways. This study provides new clues for revealing the complicated differential deposition mechanism of body fat in diverse tissues in chickens.

Methods

Animals and Ethics Statement

Three BJY chickens were obtained from the Institute of Animal Sciences, CAAS (Beijing, China), which were raised under the same recommended environmental and nutritional conditions. Animal experiments were approved by the Science Research Department (in charge of animal welfare issues) at the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (CAAS) (Beijing, China). Three birds were individually euthanized by carbon dioxide anesthesia and exsanguination by severing the carotid artery at 10 days of age, the pectoralis major and abdominal fat tissues were excised to use the cell isolation.

Preadipocyte acquisition

The mature adipocytes from the pectoralis major and abdominal fat tissue were respectively isolated as previous method, and then the preadipocytes were obtained with the dedifferentiation treatment as previous method [16]. The main protocol as follows:

The abdominal fat tissue and pectoralis major of three chickens were collected, and then washed with phosphate-buffered saline (PBS) containing 1% penicillin- streptomycin (Gibco, Thermo Fisher Scientific Inc., Shang hai, China). The abdominal fat tissue and pectoralis major from the same chicken were recorded for the one-to-one correspondence of cell samples in subsequent experiments. After removal of the blood vessels and connective tissue, the samples were finely minced to 1 mm3 with scissors, and then digested in DMEM/F12(1:1) medium (Gibco, Thermo Fisher Scientific Inc., Shang hai, China) contained 0.1% Type I collagenase (Sigma-Aldrich, Shanghai, China) in a water bath with continuous shaking at 37 °C for 60 min. After terminated digestion and filtered, the cell suspension was centrifuged with 600 g for 15 min. The top layer containing mature adipocytes was collected and placed to a 25 cm^2 cell culture flask, which was inverted and completely filled with DMEM/F12(1:1) medium containing 10% FBS. The floating mature adipocytes would adhere to the bottom of the flask incubate in a 37 °C incubator with 5% CO_2. After 3 days, the mature adipocytes would gradually converse to preadipocytes by releasing the lipid by exocytosis, and the medium was replaced and the flask was re-inverted so that the preadipocytes were on the bottom to proliferate massively. Up to 15 days, the confluence of preadipocytes would reach 80%-90%, cells were sub-cultured.

Preadipocytes culture and treatment

The medium was changed every 3 days. After reaching 80% confluence, cells were passaged, and the second passage (P2) preadipocytes by the dedifferentiation of mature adipocytes from the pectoralis major (DIMFP) and abdominal fat tissue (DAFP) were used for further experiments. Both of DIMFP and DAFP were respectively plated in 24-well or 100-mm dishes, and cells were collected for further RNA extraction (in 100-mm dishes) and used for Oil-red-O staining assay cells (in 24-well) after 2 days with 100% confluence.

Oil-red-O staining assay

The cellular lipid content of DIMFP and DAFP in 24-well were determined by oil-red-O staining. Staining procedure was conducted as follows: cell medium was discarded, washed 3 times with PBS, and then fixed with 4% formalin for 30 min. After fixed, the cells were washed 3 times with PBS and stained with oil-red-O (WuHan AmyJet Scientific Inc., Wuhan, China) for 60 min. Subsequently, the oil-red-O was discarded and washed 3 times. After water evaporated, isopropanol was added to extract the oil-red-O for 10 min, and then the absorbance values of the solutions were measured by the Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc., Shang hai, China) at 510 nm.

RNA Extraction and Identification

Total RNA was extracted from DIMFP and DAFP in 100-mm dishes, using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of RNA was detected by 1.5% gel electrophoresis, and RNA concentration was determined by NANODROP2000 spectrophotometer (Thermo Fisher Scientific Inc., Shang hai, China). The OD260/280 values of all samples were limited to the range of 1.8 to 2.0. The RNA samples were subsequently used for gene expression profiling.

Gene Expression Profiling

Based on ultra-high-throughput sequencing (HiSeq2500; Illumina, San Diego, CA, USA), gene expression profiling was undertaken by Berry Genomics (Beijing, China). Raw data were converted to FASTQ files using bcl2fastq (Illumina). Clean reads were generated by removing reads with adapter and low-quality sequences and mapped to the reference chicken genome and genes (Gallus gallus, Galgal6; available at https://www.ncbi.nlm.nih.gov/assembly/GCF_000002315.6) using TopHat 1.3.2 (https://ccb.jhu.edu/software/tophat). Gene expression levels were calculated using the RPKM method, as described by Mortazavi et al [39]. Differentially expressed genes (DEGs) between the DIMFP and DAFP were analyzed using the edgeR R package. DEGs were screened by the following criteria: $|\log2\ FC| \geq 1.0$, with $FDR < 0.05$. Based on the DEGs, clustering analysis was performed in each sample by the pheatmap package of R software. Hierarchical clustering was performed on both rows and columns, and the resulting dendrogram was saved as an image file.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analysis

Gene Ontology (GO) enrichment analysis was performed to identify the gene function classes and categories corresponding to the DEGs using the ClueGO plug-in and CluePedia plugin of Cytoscape

(https://cytoscape.org/). The significance level of GO terms enrichment was set at $P < 0.05$ as indicated. According to the results of GO enrichment analysis, DEGs related to abdominal adipose tissue metabolism were screened. The significantly enriched signaling pathways of DEGs were analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) [40]. $P < 0.05$ was considered to be indicative of statistical significance.

Real-time quantitative polymerase chain reaction

Using the same RNA samples, real-time quantitative polymerase chain reaction (qRT-PCR) was performed to confirm the results of gene expression profiling. RNA samples were reverse transcribed using TIANGEN® FastQuant RT Kit (Tiangen, Beijing, China), and specific primers were designed placing at or just outside of exon/exon junctions using Primer 5.0 software dependent on GeneBank sequences (Additional file 5:Table S5).

Samples were amplified using the real-time PCR Detection System ABI 7500 (Applied Biosystems, Carlsbad, California, USA). The PCR mixture contained 10 μL of 2 × iQ™ SYBR Green Supermix, 0.5 μL (10 mmol) of each primer, and 1 μL of cDNA, along with ddH_2O for a total volume of 20 μL. After initial denaturation for 30 s at 95 °C, amplification was performed for 40 cycles (95 °C for 5 s and 60 °C for 32 s). The actin beta (β-actin) expressions were used as the normalization control by the $2^{-\triangle\triangle Ct}$ method to determine fold-changes in gene expression [41]. A melting curve was constructed to verify the single amplified PCR product. Samples were assayed in triplicate, with standard deviations of cycle threshold values did not exceed 0.5 on a within-run basis. Correlations between relative abundance from qRT-PCR and gene expression profiling data were also calculated.

Statistical analysis

Three comparison replicates (DIMFP vs DAFP) of the cell experiment were set according to the one-to-one correspondence of cell samples from the abdominal fat tissue and pectoralis major of the same chicken. All data are presented as the means ± SEM, for three replicates of the experiment. Statistically significant differences between the two culture conditions were tested by independent-samples t-tests using SAS 9.2 software. $P < 0.05$ (*) or $P < 0.01$ (**) was considered to be significant. All figures were constructed using GraphPad Prism version 5.02 (GraphPad Software Inc, La Jolla, CA).

Abbreviations

BP: Biological Process; CC: Cellular component; CEBP: CCAAT enhancer binding protein; CIDEC: Cell death inducing DFFA like effector c; DEG: Differentially expressed genes; DIMFPs: Dedifferentiated intramuscular preadipocytes; DAFPs: Dedifferentiated abdominal preadipocytes; DGK: Diacylglycerol kinase; ELOVL: Elongase of very long-chain fatty acids-like; FABP: Fatty acid binding protein; FADS: Fatty acid desaturase; IMF: Intramuscular fat; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LPL: Lipoprotein lipase; MAPK: Mitogen- activated protein kinase; MF: Molecular Function; MOGAT: Monoacylglycerol O-acyltransferase; PBS: Phosphate-buffered saline; PLIN: Perilipin; PPAR:

Peroxisome proliferators-activated receptors; qRT-PCR: Quantitative Real-time Polymerase Chain Reaction; RBP: Retinol binding protein; RXR: Retinoid X receptor; SCD: Stearoyl-CoA desaturase; Wnt: Wingless/Int.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

ZM and NL performed the study, analyzed the data, and drafted the manuscript. JL performed the study. LL drafted the manuscript. HXC, HX and HMK contributed to the design of the study, and modifying the manuscript. GPZ and LH designed the study and was in charge of the overall project. All authors submitted comments on drafts, and read and approved the final manuscript.

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Availability of data and materials

All the data obtained in the current study have been presented in this article. The RNA-Seq sequence raw data-set supporting the results of this study have been deposited at the National Center for Biotechnology Information (NCBI).

Ethics approval and consent to participate

This study was conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). All experimental protocols were approved by the Science Research Department (in charge of animal welfare) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

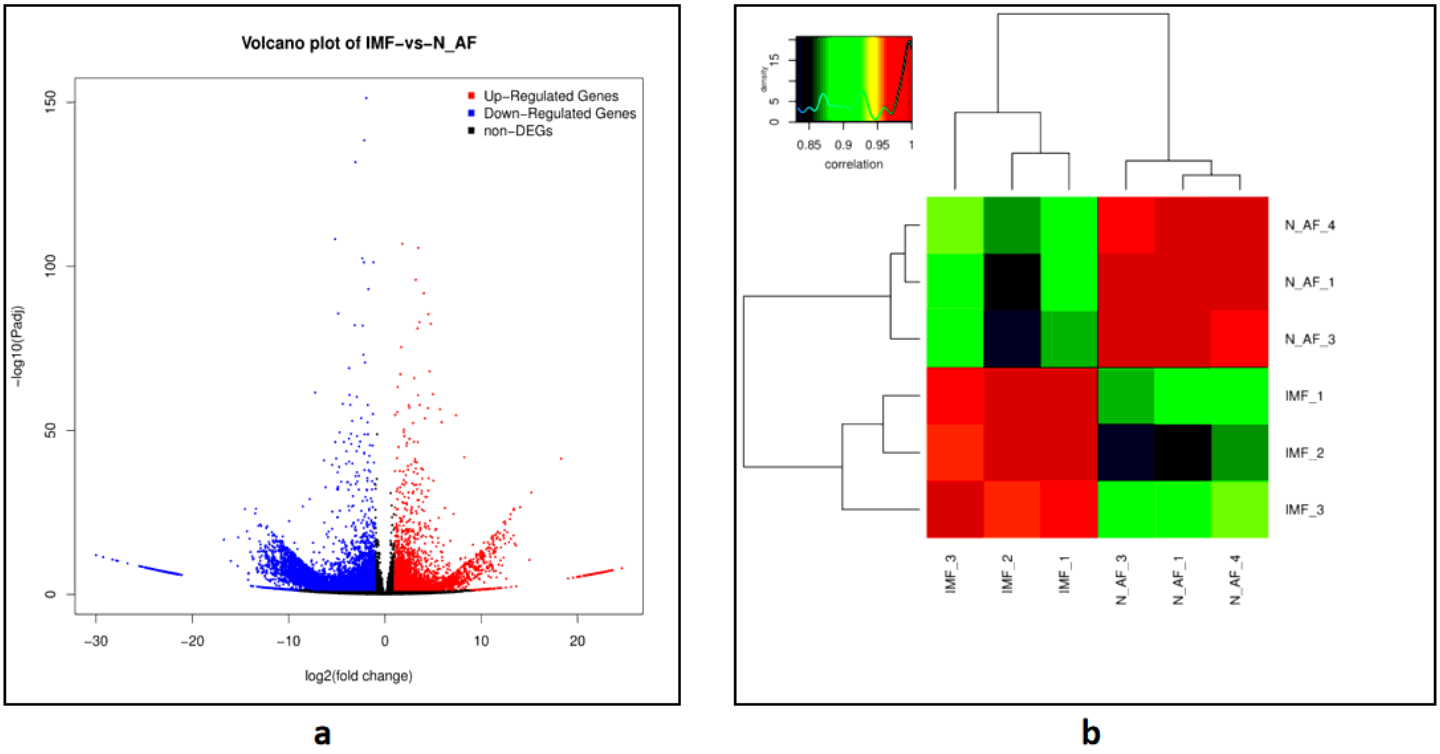

Figure 1

Volcano plot and cluster analysis of differentially expressed genes (DEGs). (a) Volcano plot. Red dots (UP) represent significantly up-regulated genes (log2 FC ≥ 1.0, FDR < 0.05); green dots (DOWN) represent significantly down-regulated genes (log2 FC ≤ −1.0, FDR < 0.05); black dots (NO) represent DEGs below the level of significance; (b) based on 3486 known DEGs in DIMFP and DAFP of chickens, the cluster analysis was performed. The results show that the data in the gene expression profiling in same group were closely related.

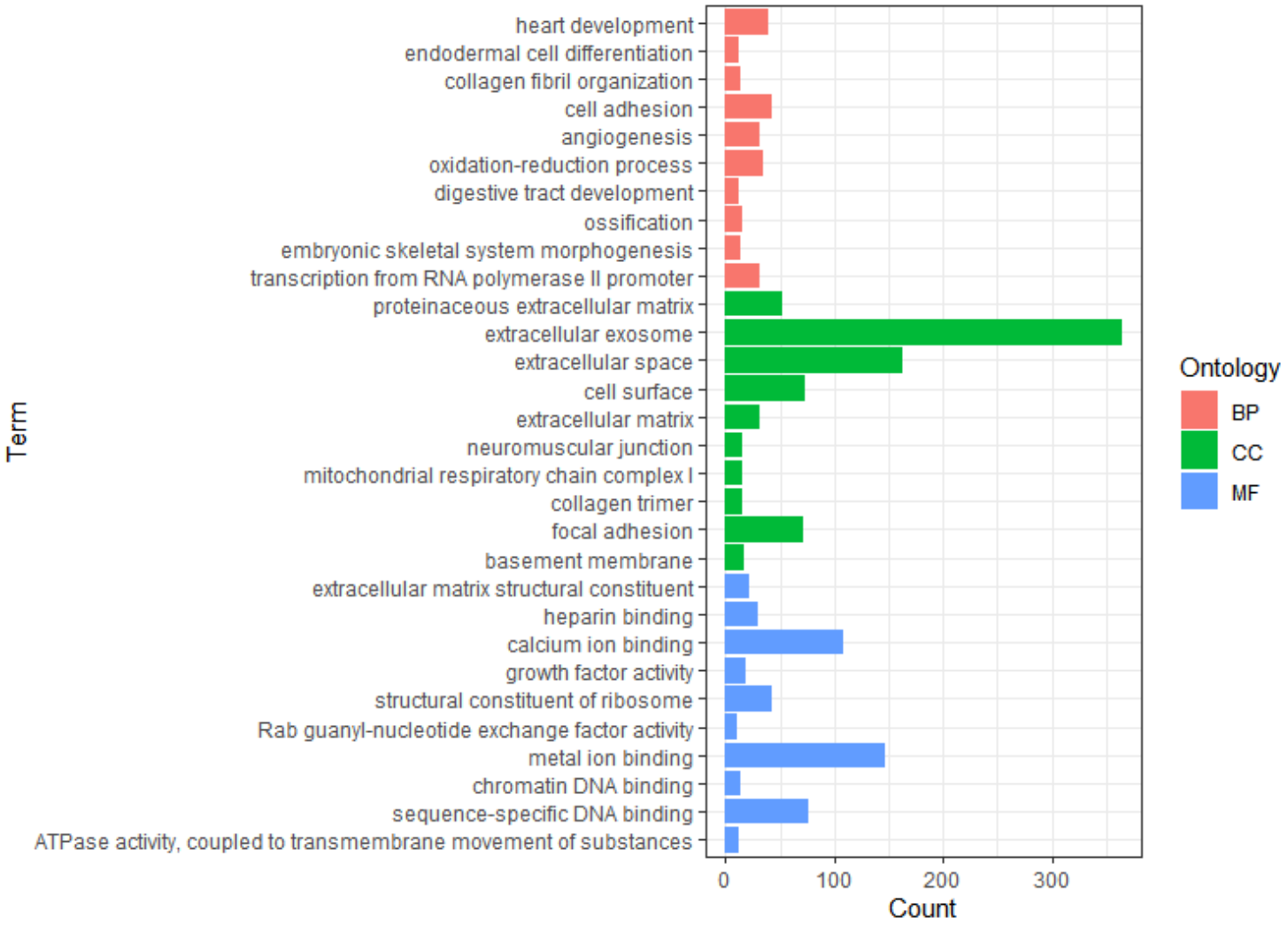

Figure 2

The list of enriched Gene Ontology (GO) terms with top 10. The enriched Gene Ontology (GO) terms were enriched (P <0.05) based on the 3486 DEGs, and the GO terms with top 10 of biological process (BP), cellular component (CC) and molecular function (MF) were listed.

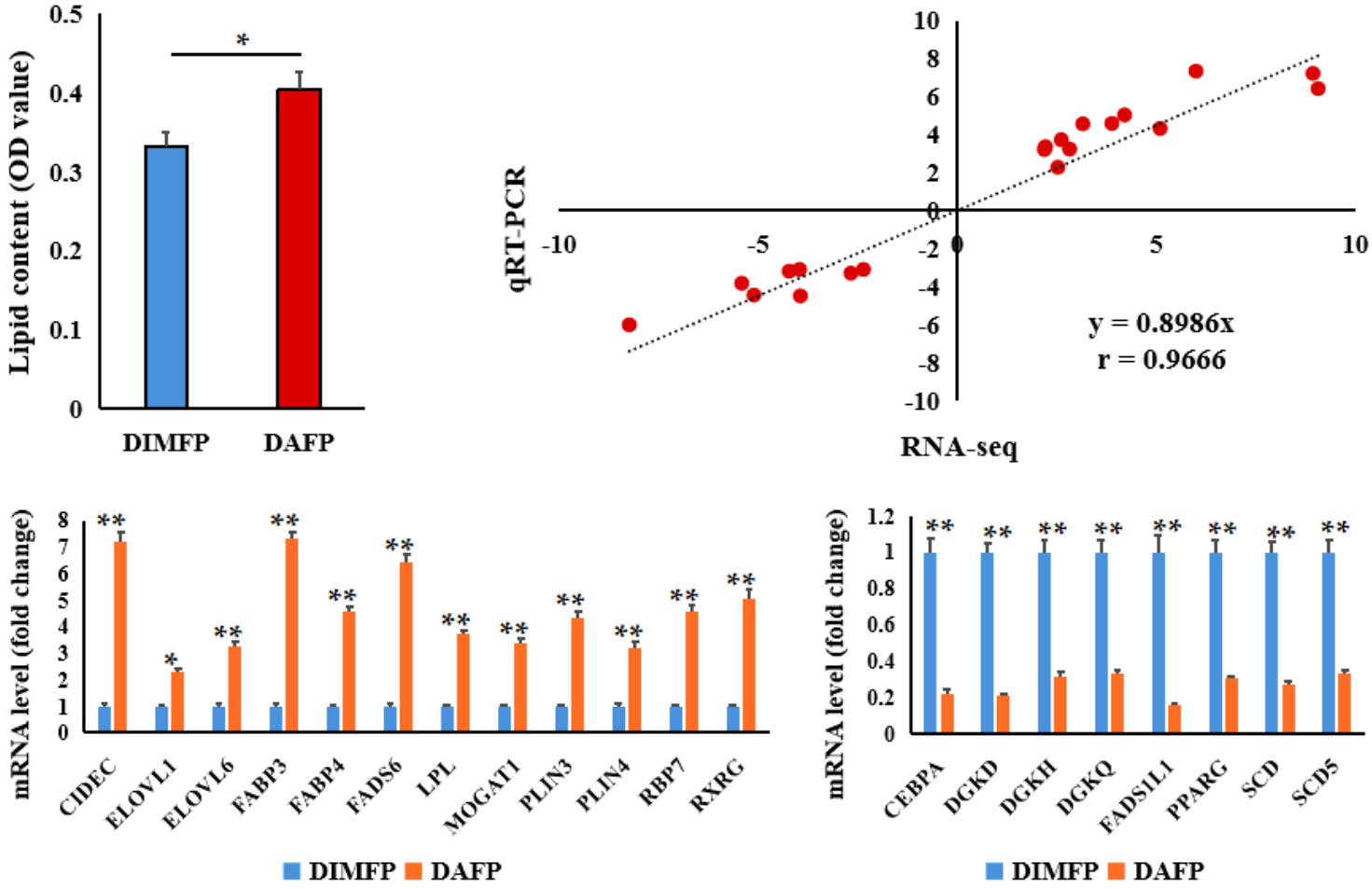

Figure 3

The difference of lipid metabolism between DIMFPs and DAFPs of chicken. (a). lipid content was increased in the DAFPs than in the DIMFP after two days with 100% confluence. Data are presented as means ± SEM (n = 3; * P < 0.05); (b). Correlation analysis of gene expression profiling and Real-time quantitative polymerase chain reaction (qRT-PCR) results by Spearman rank correlation in DIMFPs and DAFPs. A high correlation coefficient (r =0.9666, P < 0.05) was detected, which indicates that the gene expression profiling data are reliable. n = 20; (c) and (d). The qRT-PCR verification of DEGs detected by gene expression profiling. The expression levels of DEGs related to lipid metabolism determined by qRT-PCR in the DIMFPs and DAFPs. Each of these DEGs were up-regulated or down-regulated significantly (P < 0.05) in DIMFPs and DAFPs. Data are presented as means ± SEM (n = 3; * P < 0.05, ** P < 0.01);

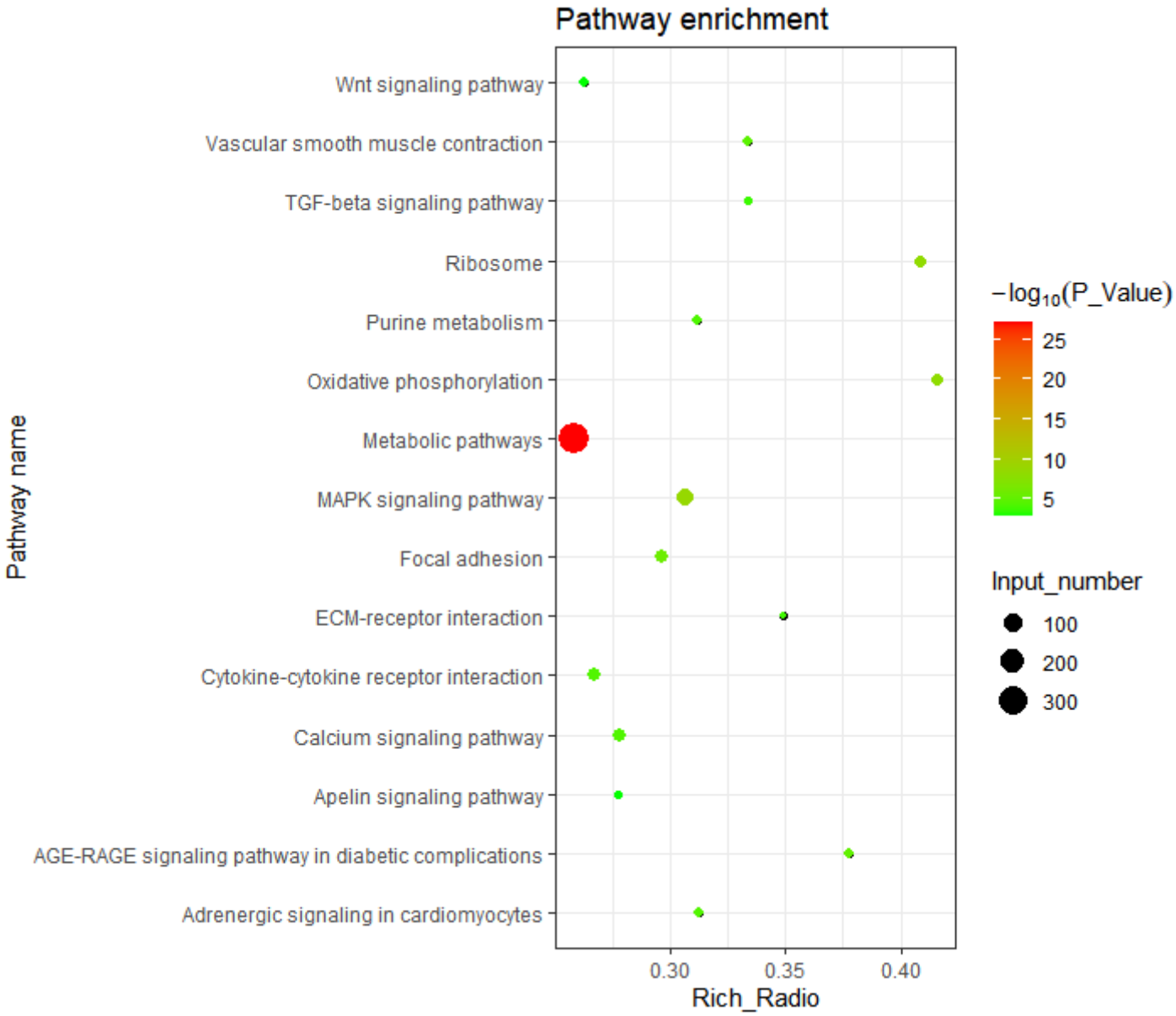

Figure 4

The list of enriched pathways with top 15 based on the 3486 DEGs. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis showed that well-known pathways (MAPK-, TGF beta-, Wnt-, Calcium-, PPAR signaling pathway) on lipid metabolism pathways were enriched, and the enriched pathways with top 15 were screen (adjusted $P < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile10KEGGPERMISSION.pdf
- Additionalfile9FigureS4.png

- Additionalfile8FigureS3.png
- Additionalfile7FigureS2.png
- Additionalfile6FigureS1.png
- Additionalfile5TableS5.xls
- Additionalfile4TableS4.xls
- Additionalfile3TableS3.xls
- Additionalfile2TableS2.xls
- Additionalfile1TableS1.xls