

Effect of Macleaya cordata extract on the growth performance and growth-related expression gene pathways in rats was investigated by liver transcriptome technology

Yong Wu (✉ wuyong712@126.com)

Hunan Agricultural University

Rongrong Wu

Hunan Agricultural University

Xiang Li

Hunan Canzoho Biological Technology Co. Ltd

Gao-feng Liu

Hunan Canzoho Biological Technology Co. Ltd

Li-Xia Shen

Hunan Agricultural University

Chong-yin Huang

Hunan Agricultural University

Yu-Hang Cao

Hunan Agricultural University

Xiong Peng

Hunan Agricultural University

Zhao-Ying Liu

Hunan Agricultural University

Zhi-Liang Sun

Hunan Agricultural University

Yi-Song Liu

Hunan Agricultural University

Research Article

Keywords: Macleaya cordata extract, rat, growth promotion, liver transcriptome

Posted Date: January 24th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2499866/v1>

Abstract

Macleaya cordata is a perennial upright herb belonging to the family Papaveraceae that represents a natural and low-residue product that has good effects in the prevention and treatment of livestock and poultry diseases and improves production performance; moreover, drug resistance to *Macleaya cordata* is difficult to establish. However, the specific mechanism of action is not clear. Therefore, this study was performed to clarify the molecular mechanism through transcriptome technology. Thirty-six specific pathogen free (SPF) male Sprague Dawley (SD) rats were divided into 3 groups of 12 rats each: high and low dose groups of 0.5 mg/10 g bw.d and 0.05 mg/10 g bw.d, respectively, and a blank group administered normal saline. Three livers from each group were collected for transcriptome sequencing, and Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, and pathway enrichment analyses of differentially expressed genes (DEGs) were performed. Transcriptome sequencing results revealed 104 upregulated and 354 downregulated genes in the high-dose group and 116 upregulated and 46 downregulated genes in the low-dose group compared with the blank group. The GO analysis showed that the DEGs were mainly associated with ATP metabolism, molecular transmembrane transport, protein decomposition and regulation, and nitrogen metabolism. The KEGG pathway enrichment analysis showed that the DEGs were mainly involved in cell proliferation and differentiation, amino acid metabolism, lipid transport, purine metabolism, and gluconeogenesis. Eight differentially expressed genes (RAD15D, BMP7, NFATC4, HDC, RPS6, RPS25, MRP133, and COX6C) verified by qRT-PCR were consistent with the trend of transcriptional sequencing.

1. Introduction

The use of antibiotics in animal husbandry has led to a series of problems, such as veterinary drug residues in the environment and negative effects to human health; therefore, corresponding measures have been taken worldwide to control or stop the use of antibiotics. The Infectious Diseases Society of America (IDSA) has issued an antimicrobial stewardship program (ASP) specifically aimed at establishing antimicrobial stewardship (Liu et al. 2014) to control the use of antibiotics. In 1997, the United Kingdom set up a 25 people working group and provided a working report with specific guidance on the use of antibiotics in all walks of life, including medicine and health, veterinary medicine, and agriculture. Denmark, as a large country for agriculture and animal husbandry, banned the use of antibiotic feed additives as early as 2000. Some Asian countries have begun to take corresponding measures to control the use of antibiotics. Since July 1, 2020, feed manufacturers in China have stopped producing commodities containing growth-promoting pharmaceutical feed additives (except for Chinese traditional medicine) (Lv 2021). Japan ensures the safety of animal products by strictly regulating the type of antibiotics used in livestock and poultry.

Macleaya cordata is a perennial upright herb of the family Papaveraceae that is mainly distributed in China, Japan, and Russia (Kosina et al. 2010; Zeng 2017). Alkaloids are the main active ingredients of *Macleaya cordata*, with the highest concentration found in the fruit, followed by the leaves, roots, and stem (Zhang et al. 2020). The main alkaloids are sanguinarine (SG), chelerythrine (CHE), protopine (PRO)

and allocryptopine (ALL). Among them, the contents of SG and CHE, which are the main pharmacodynamic components, were the highest (Liu et al. 2017). *Macleaya cordata* extract is believed to be an effective natural additive for swine, cattle, poultry, and even fish nutrition (Vieira et al. 2008). The extract of this plant is blended with intact aerial parts and a fraction of quaternary benzo [c] phenanthridine alkaloids (QBAs), primarily sanguinarine (SG) and chelerythrine (CH) (Yang 2014), and standardized to 1.5 % w/w SG (Dvořák et al. 2006).

At present, many scholars have studied the extracts of *Macleaya cordata* and found that they promote growth performance and have anti-inflammation, anti-bacterial, anti-parasitic (Xing 2021), anti-viral, and anti-tumor effects (Zeng et al. 2019). Zdarilova et al. (Zdarilova et al. 2008) fed rats a diet supplemented with *Macleaya cordata* extract for 90 days and found that the body weight of the experimental group increased significantly. Kantas et al. (Kantas et al. 2005) showed that adding *Macleaya cordata* extract to feed can increase the body weight and daily gain (ADG) of piglets and promote their growth. Montagne et al. (Montagne et al. 2007) found that the expression of ZO-1 and Clann-1 is increased in piglets after ingestion of *Macleaya cordata* extract, and these changes help prevent allergens and toxic substances from entering the intestine and improves the immune defense system of the body. Liu Gang et al. (Liu et al. 2016) performed an experiment with a control group, antibiotic group (200 mg/kg mucilage), and *Macleaya cordata* extract additive group and found a significant reduction in diarrhea score in the *Macleaya cordata* extract additive group, which proved that *Macleaya cordata* extract could enhance the intestinal barrier function of growing piglets and could be used as an alternative to antibiotics. Bavarsadi et al. (Bavarsadi et al. 2021) supplemented sanguine in the diet of laying hens and found that it improved the feed conversion rate, liver health index, systemic antioxidant capacity, and growth performance of laying hens. Lee et al. (Matulka et al. 2014) performed feed supplementation with *Macleaya cordata* extract and provided this feed to broilers continuously for 35 days after birth, and a statistical analysis of the live weight (LW), feed intake (FI), and average daily feed intake (ADG) showed that supplementation with 50 mg/kg sangrovit (containing 1.5 % of sanguine and 0.75 % of chelidonine) could improve the feed intake during the planting stage and promote growth. Tiago Ronimar Ferreira Lima et al. (Lima et al. 2020) divided 48 male Dorper×Santa Ines lambs into a traditional antioxidant group (100 IU/kg DM vitamin E and 0.1 mg/kg DM selenium) and plant extract group (*Macleaya cordata* and *Magnolia officinalis* extract) and found that the plant extract group showed greater increases in final body weight and daily weight gain, which promoted the growth of lambs. Yao Jiayun et al. (Yao et al. 2010) extracted sanguinarine from the leaves of *Macleaya cordata* extract and applied it to grass carp to determine its effects on in vitro parasite drug resistance, the result showed that the number of multifiliis on the gills in the treatment group (in 0.9 mg l⁻¹ SG) was reduced by 96.8%, in comparison to untreated group at 25°C for 48 h. These results showed that sanguine can be used as a potential source of new anti-parasite drugs.

The transcriptome serves as an important bridge between genetic information and biological function, and the generation of transcriptome sequencing (RNA-SEQ) technology can obtain all the sequence and expression information in a cell under a certain state, determine the interaction between a drug and host, and identify changes in the process of development to accurately analyze the differences in gene

structure and gene expression. Identifying the key differential genes between plants and animals and the various effects of drugs and other biological science problems (Li et al. 2020) has become an important method of studying the growth and development of livestock and poultry. In mammals, the liver is the central organ that regulates lipid synthesis and metabolism. As an important organ for digestion, absorption, and metabolism, the liver plays a key role in nutrient metabolism and immunity (Davidson et al. 2004).

Experiments by other scholars have demonstrated that *Macleaya cordata* and its extracts have growth-promoting effects on animals; however, the specific molecular mechanisms are not clear. Therefore, based on previous studies, different doses of *Macleaya cordata* extract were applied to rats via gavage for 28 d, and the results of a liver transcriptome sequencing analysis are expected to reveal the growth-promoting mechanism of *Macleaya cordata* extract in rats. A GO enrichment analysis and KEGG pathway analysis of differentially expressed genes were performed to identify the mechanisms underlying the growth promoting ability of *Macleaya cordata*. The findings provide a theoretical basis for subsequent animal experiments on the growth-promoting mechanism of *Macleaya cordata* extracts and an experimental basis for the use of these extracts as a surrogate antibody product.

2. Materials & Methods

2.1 Experimental animals and sample collection

Thirty-six healthy SPF male SD rats with body weights between 90 and 110 g were used in this experiment. The rats were fed under normal conditions for one week before the experiment, and no abnormalities were observed in the feeding, feces, or activities. Rats were randomly selected according to their weight and divided into high-dose (0.5 mg/(kg · d)), low-dose (0.05 mg/(kg·d)), and blank groups (pure water), with 12 rats in each group. The treatments were administered orally twice a day and quantified according to body weight. After 28 days of continuous intragastric administration of different doses of *Macleaya cordata* extract, the weight of the rats was measured symmetrically every 7 days and recorded, and analyzed using the SPSS 26.0 independent sample t-test. On day 28, the rats were deprived of food and water for 12 h, euthanized, and then dissected, and their livers were frozen in liquid nitrogen and stored in a -80 °C ultra-low temperature refrigerator for transcriptome sequencing.

2.2 Construction of a cDNA library and sequencing

Total RNA was extracted from rat liver tissue using the TRIzol method, and the quality of the total RNA was detected by agarose electrophoresis. Then, according to the special structure of PolyA mRNA, the obtained mRNA was purified, the mRNA with PolyA structure was enriched by Oligo (dT) magnetic beads, and the RNA was interrupted to 200–300 bp by ion interruption. Reverse transcriptase was used as a template to synthesize the first-strand cDNA, which was used as a template to synthesize the second-strand cDNA, thus forming a stable DNA double-strand structure. The resulting cDNA was subjected to the addition of an A-tail and repair of end structures, the cDNA library was amplified by polymerase chain reaction, and then fragment enrichment was carried out to form a 300–400 bp cDNA library. The libraries

were inspected using an Agilent 2100 Bioanalyzer, and double-terminal PE sequencing was performed using the Illumina HiSeq sequencing platform using second-generation sequencing technology.

2.3 Differentially expressed gene analysis

FPKM values were used to calculate the gene expression levels in the samples, and DESeq was used to screen for differential expression levels between the test and control groups. The screening condition was $|\log_2\text{FC}| > 1$, $P < 0.05$. DEGs were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses.

2.4 Verification of real-time fluorescence quantitative PCR (qRT-PCR)

The $2^{(-\Delta\Delta\text{Ct})}$ value was calculated using GAPDH as the housekeeping gene. Primer design was then carried out. CDNA in the liver tissue was used as a template for the PCR experiment, and the specificity of the product was amplified by agarose gel electrophoresis. Table 1 lists the names and primer sequences for the verified genes. A pair of appropriate primers was selected for each gene, as shown in Table 1, with GAPDH as an internal reference. Three replicates were used for each sample as controls. The relative expression level of each mRNA was determined using the $2^{(-\Delta\Delta\text{Ct})}$ method.

3. Results

3.1 Comparison of body weight between different doses of MCE

The results of the body weight analysis are shown in Table 2, with significant weight gain ($P < 0.05$) in the high- and low-dose groups and the largest weight gain in the low-dose group.

3.2 Quality control of transcriptome sequencing data

After performing computer sequencing and analysis of the rat liver tissues, 37823 million clean reads were obtained (Table 3). According to the comprehensive data, the Q20 level was greater than 96%, Q30 level 30 was greater than 92%, and other data values were also within the normal range. The results showed that the sequencing data were of high quality and could be used for subsequent analyses.

3.3 Analysis of differentially expressed genes

A volcano map of the differentially expressed genes is shown in Fig.1. The results showed that there were 104 upregulated genes and 354 downregulated genes (Fig. 1a) in the high-dose RA group compared with the blank control RC group and 116 upregulated genes and 46 downregulated genes (Fig. 1 b) in the low-dose RB group compared to the blank group RC. There were 58 upregulated genes and 26 downregulated genes (Fig. 1c) in the high-dose RA group compared with those in the low-dose RB group. The comparison between these groups revealed that the upregulated and downregulated genes were closely related to the overall function of the experimental liver transcriptome.

A Venn diagram of the three groups is shown in Fig. 2a. A total of 2996, 2934, and 3020 DEGs were obtained for FA vs FB, FA vs FC, and FB vs FC, respectively, and 2387 DEGs were found in all three groups. These results suggest that these differential genes may play an important role in rat growth.

To verify the accuracy of the biological duplication, a cluster analysis was performed on the DEGs of each group. The clustering results are shown in Fig. 2b, which show that similar genes in the same dose group were clustered together, thus demonstrating the accuracy and reliability of the sequencing results.

3.4 GO enrichment analysis of differentially expressed genes

A GO functional enrichment analysis was performed for the DEGs in each group. The results for the three groups are shown in Fig. 3a (RC vs RA), Fig. 3b (RC vs RB), and Fig. 3c (RA vs RB). Significant enrichment of 30, 30, and 19 GO items was found in the three groups, which each had 10 items categorized as biological processes (BP). The experimental results identified some important biological processes (BP) related to growth and development, such as peptide metabolism, cytoamide metabolism, cellular nitrogen compound biosynthesis, purine nucleotide metabolism, ATP metabolism, purine ribose triphosphate metabolism, and purine nucleoside triphosphate metabolism.

3.5 KEGG pathway enrichment analysis of differentially expressed genes

A KEGG pathway analysis was performed on the DEGs in the three groups. The first 20 KEGG pathways in each group are shown in Fig. 4a (RC vs RA), 4b (RC vs RB), and 4c (RA vs RB). The first 20 KEGG pathways in each group were mostly related to growth and development, including cell proliferation and differentiation, amino acid metabolism, lipid transport, purine metabolism, and gluconeogenesis. Although some of these pathways did not reach significance (corrected P-value ≤ 0.05), they still have important reference value for production and theoretical studies. In the RC vs RA, RC vs RB, and RA vs RB groups, 5, 6, and 2 KEGG pathways were significantly enriched in DEGs, respectively. Differential genes were mainly enriched in the spliceosome, ribosome, PPAR signaling pathway, oxidative phosphorylation, myocardial contraction, glycolysis/glycogenesis, DNA replication, cell cycle, and other pathways. They play an important role in studies on production and mechanisms in rats.

3.6 qRT-PCR verification results

To demonstrate whether the RNA-seq data are true and reliable, 20 genes were screened as candidate genes based on a STRING protein interaction analysis, and functional maps of different groups of differential gene expression were obtained (Fig. 5a). The transcriptomic expression data of these genes were also referenced, and eight genes were selected for fluorescence quantitative PCR validation. In this study, eight genes (*RAD15D*, *BMP7*, *NFATC4*, *HDC*, *RPS6*, *RPS25*, *MRP133*, and *COX6C*) were selected for qRT-PCR verification (Fig. 5b). The qRT-PCR results were consistent with the RNA-seq data, thus demonstrating that the RNA-seq test data are true and reliable.

4. Discussion

Studies have shown that the extract of *Macleaya cordata* can promote animal growth, improve growth performance (Luo et al. 2021), improve feed conversion rate, and reduce diarrhea rate (Zong et al. 2020). The growth and development of animals are influenced by many genes at the transcriptome level. In this study, transcriptomic techniques were used to investigate the regulatory mechanisms of the genes involved in growth promotion in rats. The experimental results showed that the liver transcriptome sequencing results of rats in the high and low dose group supplemented with *Macleaya cordata* extract were significantly different from those in the control group.

A GO enrichment analysis was performed on the differentially expressed genes in the three experimental groups (RA vs RB, RA vs RC, and RB vs RC). The results showed that maximum changes occurred in peptide metabolism, cytoamide metabolism, cellular nitrogen compound biosynthesis, purine nucleotide metabolism, ATP metabolism, purine ribose triphosphate metabolism, and purine nucleoside triphosphate metabolism.

A KEGG pathway analysis was performed for the differentially expressed genes in the three experimental groups (RA vs RB, RA vs RC, and RB vs RC), and the first 20 pathways in each group are shown in Fig. 4. The results showed that the splicing body, ribosome, PPAR signaling pathway, oxidative phosphorylation, myocardial contraction, glycolysis/glycogenesis, DNA replication, cell cycle, and other pathways were significantly enriched. At the same time, the high- and low-dose groups and the control group presented different DEGs, indicating that different doses of MCE had different effects on the liver of rats. qRT-PCR was used to verify the eight differentially screened genes, and the expression trends of these eight genes were consistent with the sequencing results, which demonstrated the reliability of this experiment and the accuracy of the sequencing results.

RNA-seq technology can be used to explore the effects of *Macleaya cordata* extract on the genes of rat liver cells and identify the genes and signaling pathways related to growth and development (Wang 2020). Therefore, in this study, transcriptome sequencing was performed on rat livers. The results showed that 16109, 16332, and 16158 differentially expressed genes were obtained in the RA vs RB, RA vs RC, and RB vs RC groups, respectively. Several common DEGs were also identified, such as *COX6C*, *HDC*, *MRPL33*, *NFATC4*, *RPS25*, *RPS6*, and *RAD51D*. Through this analysis, we also found genes related to growth and development among the enriched pathways, such as *CycD*, *SCD-1*, and *EPOR*. In addition, five important pathways were identified, thus providing a basis for further understanding the molecular mechanism of *Macleaya cordata* extract in rats.

BMP7 (bone morphogenetic protein 7) is a protein-encoding gene that plays an important role in calcium regulation and bone homeostasis in vivo by regulating mesenchymal stem cells due to its ability to induce bone and cartilaginous tissue formation (Sun et al. 2005). *BMP7* expression also promotes the differentiation of C2C12 cells through the BMP/TGF- β pathway to promote muscle development (Wang et al. 2019). Therefore, earlier encoded primordium is hydrolyzed to form homodimeric disulfide bonds, and each of these subunits can play an important role in the development of bone, kidney, and fat. This gene also has a very important collateral homologue, *BMP5*, which plays a regulatory role in various cancers to

varying degrees (Korkut et al. 2018). In the validation test of this experiment, the level of *BMP7* increased successively from the blank group to the low-dose and high-dose groups, indicating that the effect of *Macleaya cordata* extract on *BMP7* is consistent with its function.

COX6C (cytochrome C oxidase subunit 6 C) is the last enzyme in the mitochondrial electron transport chain that drives oxidative phosphorylation, thus enabling electron transfer from reduced cytochrome C to oxygen molecules, and it plays an indispensable role in cellular oxidative phosphorylation (Tian et al. 2021). *COX6C* can cooperatively transfer the derived electrons from NADH and succinate to oxygen molecules, thus generating an electrochemical gradient on the intima to drive transmembrane transport and ATP synthase (Hashad et al. 2016). The results of the validation test showed that *COX6C* expression in the high- and low-dose groups was higher than that in the blank group. To a certain extent, *Macleaya cordata* extract can promote the expression of *COX6C*, which is beneficial for the growth of rats. However, the validation test was higher in the low-dose group than in the high-dose group compared with transcriptome sequencing, which requires further discussion and research.

HDC (histidine decarboxylase) can convert histidine to histamine, which acts as a neurotransmitter and plays an important role in the function of a variety of brain activities, and astrocytes can transport it (Yoshikawa et al. 2016). Stimulation by activating the H_1 receptor causes the hypothalamus to release more histamine, resulting in reduced food intake and weight loss (Díaz et al. 2019). The above findings show that the liver is the main metabolic site, which can affect the amount of food intake through neural reflection. The expression of *HDC* increased successively from the blank group to the low-dose group and finally to the high-dose group, indicating that the extract of *Macleaya cordata* can cause more significant enrichment of this gene, thus further promoting the growth of rats.

MRPL33 (mitochondrial ribosomal protein L33) can participate in the translation process in mitochondria and plays a certain role in the protein synthesis process in mitochondria, which is necessary for the normal operation of organelles (Cheong et al. 2020). Bioinformatics analysis showed that the overexpression of *MRPL33-L* and *MRPL33-S* played a key role in transcription, signal transduction, and apoptosis, and these signaling pathways showed an opposite effect in the chemical reaction of gastric cancer to epirubicin, which was conducive to the resolution of drug resistance in gastric cancer patients (Li et al. 2019). On the one hand, a growth promotion effect was observed for the extract of *Macleaya cordata*, while on the other hand, increases in the dose did not improve the effect. Moreover, an optimal peak value was observed.

NFATC4 (nuclear factor of activated T cell 4) is a regulated transcription factor involved in multiple biological processes, and aberrantly activated *NFATC4* participates in modulating the initiation, proliferation, invasion, and metastasis of various cancers (Zhong et al. 2022). It can also be involved with *NFATC3* in the development of the embryonic heart and in the energy metabolism of mitochondria required for its function, which can change the shape of the heart. It can bind to and simultaneously activate the BACE1 / β -secretase 1 promoter, thereby participating in the proteolytic process of amyloid precursor protein regulation (Mei et al. 2015). p38 MAP increases the expression of PPAR γ by

phosphorylating several residues in the NFAT homology domain of *NFATC4*, which contributes to adipocyte formation and plays a direct role in adipocyte differentiation (Yang et al. 2002). After drug treatment, the expression of the *NFATC4* gene was significantly increased in the blank, high-dose, and low-dose groups, and it could be seen from the KEGG pathway diagram that the decrease in the adrenal ketone hormone content in the body of the rats could lower blood pressure and form fat cells to promote healthy growth of the rats.

Rad51D is a member of the *Rad51* family. This protein-coding gene translates into a protein complex that promotes homologous pairing between single- and double-stranded DNA, homologous recombinant repair (HRR) pathways that can participate in the DNA replication process, and double-stranded DNA breaks occur during the overrun or are induced by DNA damage agents (Baldock et al. 2019). Because *RAD51D* is an important part of homologous recombination, if this gene is defective, it will have a huge negative impact on cell mitosis; therefore, this gene plays an indispensable role in various biological processes of DNA (Wyatt et al. 2018). Through transcriptome high-throughput sequencing and quantitative fluorescence PCR experiments, we found that the expression level of *RAD51D* in the low-dose and high-dose groups was significantly higher than that in the blank group. These results indicated that *Macleaya cordata* extracts promoted DNA mitosis, homologous pairing, and other biological processes in the cells, which ensured the growth and development of rats.

RPS6 (ribosomal protein S6) is one of the main substrates for the lipid kinase reaction. Owing to its special structure, *RPS6* has five C-terminal serine residue subsets that are phosphorylated by different protein kinases and selectively recognize different mRNAs to control cell proliferation (Meyuhas. 2015). It was found that the expression level in the high-dose group was the highest, followed by the low-dose group and the blank group. According to the KEGG analysis, this gene is mainly involved in the HIF-1 signaling pathway, and inhibition of HIF-1 can improve the severity of osteoporosis (Zhong et al. 2017). As the expression level of the gene is downregulated, it can be inferred that the extract of *Macleaya cordata* can increase the inhibitory effect on the HIF-1 signaling pathway through the influence of the *RPS6* gene. Promote bone development and growth in rats.

RPS25 (ribosomal protein S25) encodes a cytoplasmic ribosomal protein that is part of the S25E family and is present in the cytoplasm, similar to other ribosomal genes. *Rps25* is directly involved in the translation control of proteins, and after *RPS25* knockout, the target ribosome is altered, showing virus and toxin resistance phenotypes, and these phenotypes cannot be saved by functional cDNA expression (Johnson et al. 2020). Transcriptome sequencing and qRT-PCR validation tests showed that the expression level of the blank group was the lowest, followed by the low-dose group and the highest high-dose group, which indicated that the influence of the *RPS25* gene could enhance protein translation and thus promote the growth of rats.

In addition to the eight common DEGs mentioned above, we identified eight DEGs related to growth and development in two pathways. Animal growth, whether it is the development of each organ or system, cannot be separated from the basic biological processes of cell division and differentiation. According to

the KEGG enrichment pathway, it can be seen that after the intragastric administration of the extract, many pathways conducive to growth were enriched. One such pathway is the JAK-STAT signaling pathway, which is a cytokine-stimulatory signaling pathway (Fig. 6a). The JAK-STAT signaling pathway is involved in the pathogenesis of inflammation and immune diseases.

In this signaling pathway, the *CyCD* gene is upregulated, which belongs to one of the cyclins, plays the role of a CDK kinase regulator, and contributes to the time coordination during mitosis of each cell. The RBF/E2F pathway enables mitosis to proceed in an orderly and regular manner. It also plays an important role in cell proliferation (Romero-Pozuelo et al. 2020; Frei et al. 2005). *EPOR* (erythropoietin receptor) is also an upregulated gene that enhances the activation of JAK2 tyrosinase. In mice, erythropoietin can also inhibit the NLRP3 inflammasome through *EPOR* to control inflammation (Cao et al. 2020). In other words, the upregulation of this gene can also effectively prevent the occurrence of inflammation, promote mitosis, and make cells proliferate and differentiate, thus promoting the growth and development of rats.

The PPAR (peroxisome proliferator-activated receptor) pathway plays a key role in glucose and lipid metabolism, mainly through peroxisome proliferator-activated receptors acting as ligand-induced transcription factors (Mirza et al. 2019). In the PPAR pathway, the receptor is induced by transcription factors and acts on the target gene. The target gene is upregulated or downregulated to varying degrees in rats after intragastric administration of the extracts, and the biological effect also changes to some extent.

In the pathway obtained in this experiment (Fig. 6b), the SCD-1 (stearyl coenzyme A desaturase) gene is upregulated. This gene can add a double bond to the saturated fat acyl-CoA substrate, catalyze the cis-double bond at $\Delta 9$ to insert fatty acyl-CoA substrate, and convert saturated fatty acids to monounsaturated fatty acids. It also plays an important role in lipid biosynthesis, regulating the expression of genes related to fat formation, the oxidation of mitochondrial lipoic acid, and the dynamic energy balance of the body and contributes to the biosynthesis of membrane phospholipids, cholesterol esters, and triglycerides. PPAR- γ is a key regulator of adipocyte differentiation and glucose homeostasis. It activates the nuclear receptors of peroxidase proliferators, binds to the specific PPAR response element (PPRE) of DNA, and regulates the transcription of its target genes(such as acyl-coenzyme A oxidase). Five genes, *Apo-A II*, *Apo-C III*, *ACBP*, *FABP1*, and *AP2*, were downregulated, and they were the target genes involved in lipid transport, fatty acid transport, and fatty acid oxidation. Therefore, these significantly upregulated and downregulated genes play an important role in promoting rat growth.

5. Conclusions

The function and metabolism of the rats changed after continuous administration of *Macleaya cordata* extract for 28 days. According to the KEGG and GO analyses, these extracts can promote the proliferation and differentiation of rat cells and the metabolism of nutrients, such as fat, sugar, and amino acids. This study provides a basis for a preliminary understanding of the mechanism underlying growth promotion by the extract of *Macleaya cordata* in rats and provides a reference for subsequent experiments focusing

on the differentially enriched genes and gene pathways to further and comprehensively understand the associated growth mechanism.

Declarations

Author Contributions:

Zhao-Ying Liu, Yi-Song Liu, Zhi-Liang Sun: Conceptualization, Methodology

Yong Wu, Li-Xia Shen, Chong-yin Huang, Yu-Hang Cao, Xiong Peng: Investigation and Validation

Yi-Song Liu, Rong-Rong Wu, Xiang Li, Gao-Feng Liu: Formal analysis and Data Curation

Yong Wu, Rong-Rong Wu, Zhi-Liang Sun: Writing-Reviewing and Editing

Funding: This study was financially supported by the National Natural Science Foundation of China (Grant No. 31402255).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, all rats in the experiment came from Hunan Slack Jingda Laboratory Animal Co. LTD and were raised in the animal facilities of College of Veterinary Medicine, Hunan Agricultural University. The experimental scheme and process met the local ethical review standards.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data were uploaded on March 26, 2022 and will be made publicly available on April 30, 2024 as the paper is unpublished.

Acknowledgments: We thank all members for their generous participation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Baldock RA, Pressimone CA, Baird JM, Khodakov A, Luong TT, Grundy MK, Smith CM, Karpenshif Y, Bratton-Palmer DS, Prakash R et al (2019) RAD51D splice variants and cancer-associated mutations reveal XRCC2 interaction to be critical for homologous recombination. *DNA Repair (Amst)* 76, 99–107. <https://doi.org/10.1016/j.dnarep.2019.02.008>.
2. Bavarsadi M, Mahdavi AH, Ansari-Mahyari S, Jahanian E (2021) Sanguinarine improved nutrient digestibility, hepatic health indices and productive performance in laying hens fed low crude protein diets. *Vet Med Sci* 7, 800–811. <https://doi.org/10.1002/vms3.436>.
3. Cheong A, Archambault D, Degani R, Iverson E, Tremblay KD, Mager J (2020) Nuclear-encoded mitochondrial ribosomal proteins are required to initiate gastrulation. *Development* 147.

<https://doi.org/10.1242/dev.188714>.

4. Cao F, Tian XY, Li ZW, Lv Y, Han J, Zhuang R, Cheng BH, Gong YQ, Ying BY, Jin SW et al (2020) Suppression of NLRP3 inflammasome by erythropoietin via the EPOR/JAK2/STAT3 pathway contributes to attenuation of acute lung injury in mice. *Front Pharmacol* 11, 306. <https://doi.org/10.3389/fphar.2020.00306>.
5. Dvořák Z, Sovadinová I, Bláha L, Giesy JP, Ulrichová J (2006) Quaternary benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine do not affect transcriptional activity of aryl hydrocarbon receptor: analyses in rat hepatoma cell line H4IIE.luc. *Food Chem Toxicol* 44, 1466–1473. <https://doi.org/10.1016/j.fct.2006.04.016>.
6. Díaz NF, Flores-Herrera H, García-López G, Molina-Hernández A (2019) Central histamine, the H3-receptor and obesity therapy. *CNS Neurol Disord Drug Targets* 18, 516–522. <https://doi.org/10.2174/1871527318666190703094846>.
7. Frei C, Galloni M, Hafen E, Edgar BA (2005) The *Drosophila* mitochondrial ribosomal protein mRpl12 is required for cyclin D/Cdk4-driven growth. *EMBO J* 24, 623–634. <https://doi.org/10.1038/sj.emboj.7600523>.
8. Hashad D, Elgohry I, Dwedar F (2016) Nuclear respiratory Factor-1 (NRF-1) gene expression in chronic kidney disease patients undergoing hemodialysis and mitochondrial oxidative dysregulation. *Clin Lab* 62, 2149–2154. <https://doi.org/10.7754/Clin.Lab.2016.160329>.
9. Johnson AG, Flynn RA, Lapointe CP, Ooi YS, Zhao ML, Richards CM, Qiao W, Yamada SB, Couthouis J, Gitler AD et al (2020) A memory of eS25 loss drives resistance phenotypes. *Nucleic Acids Res* 48, 7279–7297. <https://doi.org/10.1093/nar/gkaa444>.
10. Kosina P, Gregorova J, Gruz J, Vacek J, Kolar M, Vogel M, Roos W, Naumann K, Simanek V, Ulrichova J (2010) Phytochemical and antimicrobial characterization of *Macleaya cordata* herb. *Acta phytotaxonomica sinica. Fitoterapia* 81, 1006–1012. <https://doi.org/10.1016/j.fitote.2010.06.020>
11. Kantas D, Papatsiros VG, Tassis PD, Athanasiou LV, Tzika ED (2015) Effect of a natural feed additive (*Macleaya cordata*), containing sanguinarine, on the performance and health status of weaning pigs. *Anim Sci J* 86, 92–98. <https://doi.org/10.1111/asj.12240>.
12. Korkut A, Zaidi S, Kanchi RS, Rao S, Gough NR, Schultz A, Li X, Lorenzi PL, Berger AC, Robertson G et al (2018) A Pan-cancer analysis reveals high-frequency genetic alterations in mediators of signaling by the TGF- β superfamily. *Cell Syst* 7, 422–437.e7, abstract 3134:A. <https://doi.org/10.1016/j.cels.2018.08.010>.
13. Liu YN, Zhang YJ, Hu MM, Li PJ (2014) Discussion on foreign antibiotic management strategy. *Chin J New Drugs* 23, 1836–1842.
14. Liu G, Guan GP, Fang J, Martínez Y, Chen S, Bin P, Duraipandiyan V, Gong T, Tossou MC, Al-Dhabi NA et al (2016) *Macleaya cordata* extract decreased diarrhea score and enhanced intestinal barrier function in growing piglets. *BioMed Res Int* 1069585. <https://doi.org/10.1155/2016/1069585>.
15. Liu XB, Huang JLu, Zheng YJ, Liu W, Tang ZS, Zeng JG (2017) Comparison of alkaloids in different parts of *Macleaya cordata* and *Macleaya micordata*. *China Mod Chin Mater Med* 19, 1382–1386.

<https://doi.org/10.13313/j.issn.1673-4890.2017.10.006>.

16. Li J, Feng D, Gao CX, Zhang, YG, Xu J, Wu MH, Zhan XB (2019) Isoforms S and L of MRPL33 from alternative splicing have isoform-specific roles in the chemoresponse to epirubicin in gastric cancer cells via the PI3K/AKT signaling pathway. *Int J Oncol* 54, 1591–1600.
<https://doi.org/10.3892/ijo.2019.4728>.
17. Lima TRF, Gallo SB, Rosa AF, Silva DLES, Brochado T, Bezerra HVA, Putrino SM, Martins MB, Leme PR (2020) Effect of *Macleaya cordata* and *Magnolia officinalis* plant extracts on oxidative stress control in lambs fed a high-concentrate diet. *Asian-Australas J Anim Sci Jun*;33(6):913-920.
18. Li XY, Xia YJ, Xu L, Zhao QZ, Hu YH, Wang Q, Zhang QY (2020) Advances in transcriptome research on host infected with Classical Swine Fever Virus based on RNA-SEQ. *Chin J Vet Med* 54, 79–85.
19. Lv (2021) Discussion and research on antibiotic alternatives after prohibition in the feed industry. *Animal Husbandry and Veterinary Science (electronic version)* 02, 23-25.
20. Luo X, Yu WH (2021) Effects of *Macleaya cordata* extracts on growth performance of Weaned piglets. *Animal and Poultry Industry* 32, 2.
21. Montagne L, Boudry G, Favier C, Le Huërou-Luron I, Lallès JP, Sève B (2007) Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *Br J Nutr* 97, 45–57. <https://doi.org/10.1017/S000711450720580X>.
22. Matulka RA, Alvenslebe Sv, Morlacchin M (2014) Tolerance and residue study for standardized *Macleaya cordata* extract added to chicken feed. *Int J Poult Sci* 13, 368–373.
<https://doi.org/10.3923/ijps.2014.368.373>.
23. Meyuhas O (2015) Ribosomal protein S6 phosphorylation: four decades of research. *Int Rev Cell Mol Biol* 320, 41–73. <https://doi.org/10.1016/bs.ircmb.2015.07.006>.
24. Mei ZG, Yan PK, Tan XP, Zheng SM, Situ B (2015) Transcriptional regulation of BACE1 by NFAT3 leads to enhanced amyloidogenic processing. *Neurochem Res* 40, 829–836.
<https://doi.org/10.1007/s11064-015-1533-1>.
25. Mirza AZ, Althagafi II, Shamshad H (2019) Role of PPAR receptor in different diseases and their ligands: physiological importance and clinical implications. *Eur J Med Chem* 166, 502–513.
<https://doi.org/10.1016/j.ejmech.2019.01.067>.
26. Romero-Pozuelo J, Figlia G, Kaya O, Martin-Villalba A, Teleman AA (2020) Cdk4 and Cdk6 couple the cell-cycle machinery to cell growth via mTORC1. *Cell Rep* 31, 107504.
<https://doi.org/10.1016/j.celrep.2020.03.068>.
27. Sun DM, Hou SX, Tong YG, Du GX, Fu XB (2003) Cloning and sequence analysis of bone morphogenetic protein 7 gene. *Chin J Orthop Surg* 21, 46–48.
28. Tian BX, Sun W, Wang SH, Liu PJ, Wang YC (2021) Differential expression and clinical significance of COX6C in human diseases. *Am J Transl Res* 13, 1–10.
29. Vieira SL, Oyarzabal OA, Freitas DM, Berres J, Peña JEM, Torres CA, Coneglian JLB (2008) Performance of broilers fed diets supplemented with sanguinarine-like alkaloids and organic acids. *J Appl Poult Res* 17, 128–133. <https://doi.org/10.3382/japr.2007-00054>.

30. Wyatt MD, Reilly NM, Patel S, Rajesh P, Schools GP, Smiraldo PG, Pittman DL (2018) Thiopurine-induced mitotic catastrophe in Rad51d-deficient mammalian cells. *Environ Mol Mutagen* 59, 38–48. <https://doi.org/10.1002/em.22138>.
31. Wang YX, Liu SY, Yan YQ, Li SF, Tong HL (2019) SPARCL1 promotes C2C12 cell differentiation via BMP7-mediated BMP/TGF-beta cell signaling pathway. *Cell Death Dis* 10, 852.
32. Wang Z (2020) Effects of *Lactobacillus rhamnosus* on intestinal homeostasis and resistance to intestinal pathogens infection; *Northeast Agricultural University* 04, 87.
33. Xing WH (2021) Application of active components and extracts of *Macleaya cordata* extract in livestock and poultry production. *Feed Res* 44, 3.
34. Yang TTC, Xiong QF, Enslen H, Davis RJ, Chow CW (2002) Phosphorylation of NFATc4 by P38 mitogen-activated protein kinases. *Mol Cell Biol* 22, 3892–3904. <https://doi.org/10.1128/MCB.22.11.3892-3904.2002>.
35. Yao JY, Shen JY, Li XL, Xu Y, Hao GJ, Pan XY, Wang GX, Yin WL (2010) Effect of sanguinarine from the leaves of *Macleaya cordata* against *Ichthyophthirius multifiliis* in grass carp (*Ctenopharyngodon idella*). *Parasitol Res* 107, 1035–1042. <https://doi.org/10.1007/s00436-010-1966-z>.
36. Yang XJ (2014) Biomimetic Design, Synthesis, Biological Activity and Structure-Activity Relationship of Novel Natural QBAs Analogues and Their Derivatives. Northwest A&F University.
37. Yoshikawa T, Yanai K (2016) Histamine clearance through polyspecific transporters in the brain. *Handb Exp Pharmacol* 241, 173–187.
38. Zeng JG (2017) *Macleaya micordata* resources as a demonstration of the whole industry chain of Chinese herbal medicine research and comprehensive utilization. *Chin Med* 19, 1359–1366.
39. Zhang LM, Zhu JJ, Yang LY, Tang SX (2020) Study on the application of wave return in livestock and poultry production. *Chin J Laser Biol* 29, 113–119.
40. Zeng JG, Liu H (2019) The main functional components of *Macleaya cordata* extract and their application in animal production. *Feed Ind* 40, 6.
41. Zdarilova A, Vrublova E, Vostalova J, Klejdus B, Stejskal D, Proskova J, Kosina P, Svobodova A, Vecera R, Hrbac J et al (2008) Natural feed additive of *Macleaya cordata*: safety assessment in rats a 90-day feeding experiment. *Food Chem Toxicol* 46, 3721–3726. <https://doi.org/10.1016/j.fct.2008.09.054>.
42. Zong WL, Deng LF, Wang D, Xin N Guo L (2020) Effects of *Macleaya cordata* extract on growth performance and diarrhea rate of growing blue foxes. *Feed Expo* 12, 4.
43. Zhong QH, Zha SW, Lau ATY, Xu YM (2022) Recent knowledge of NFATc4 in oncogenesis and cancer prognosis. *Cancer Cell Int* 22, 212.
44. Zhong H, Cao C, Yang J, Huang Q (2017) Research on relationship of HIF-1 signaling pathway and postmenstrual osteoporosis. *Sichuan Da Xue Xue Bao Yi Xue Ban* 48, 862–868.

Tables

Tab 1 PCR primer information

Name of primer	Primer sequences	Length (bp)	Name of primer
GADPH	F GCCAACCCCCAATGTCTCT R:CAACCTGGTCCTCTGTGTATCCT	140	GADPH
Rad51d	F TGTCTCTGTGTGGCTGCAAA R TGGAGAGCACTTGCCTGTTT	146	Rad51d
BMP7	F CAAGGCCGTCTTCAGTACCC R CTTGTCGTGCTCCACTAGGTT	112	BMP7
Nfatc4	F GAGTACAGCAACAAGCGGGT R TCCTCCTTGAAGATCACAGGC	119	Nfatc4
Hdc	F AATCATCATGCCTGGGGTGG R CAAGCAGTTGATGGCGTCAG	118	Hdc
RPS6	F TCGTCGGTTGGGACCTAAAAG R TCCTGGGCTTCTTACCTTCTT	122	RPS6
RPS25	F GCAAGGCCAAAAAGAAGAAGTG R TCAGTCTCTCGGAGACCACA	150	RPS25
MRP133	F TGCCAAGAGCAAGTCAAAAACCT R GGGTCAGCTTCTCTCGGAGT	104	MRP133
COX6C	F ATGAGTTCCGGTGCTCTGTT R TTCAGCCACGCCAAACTTAT	132	COX6C

Table 2 Effects of continuous administration of *Macleaya cordata* extract for 28 days on body weight of male rats(

Groups	Give medicine	7 d	14 d	21 d	28 d
Control group	105.1 ± 10.2	150.3 ± 13.3	195.7 ± 14.9	236.8 ± 15.6	271.7 ± 18.5
Low dose group	106.0 ± 10.5	160.9 ± 14.0	226.8 ± 14.0*	271.1 ± 17.1*	310.2 ± 20.0*
High dose group	103.4 ± 8.0	161.7 ± 13.5	208.9 ± 17.5	249.4 ± 17.3	284.6 ± 20.2

Note: Note: Compared with blank control group * P < 0.05.

Table 3 RNA-Seq results

Sample	Q20 (%)	Q30 (%)	Clean reads	Clean reads(%)	Total mapped	Multiple mapped(%)	Uniquely mapped(%)
RA1	96.62	92.2	43704110	98.99	85.46	6.48	93.52
RA2	95.78	90.69	40574450	98.27	83.55	6.02	93.98
RA3	96.17	91.33	41290360	98.70	85.44	6.32	93.68
RB1	97.39	93.66	43084760	99.47	87.77	5.28	94.72
RB2	95.81	90.73	40739460	98.24	84.00	6.27	93.73
RB3	94.48	88.61	39151974	96.31	81.65	6.24	93.76
RC1	97.57	94.03	44218626	99.56	88.63	5.03	94.97
RC2	97.67	94.26	44918152	99.63	86.89	7.40	96.60
RC3	97.59	94.09	40553266	99.55	87.37	5.76	94.24

Note: Ra1-RA3 high-dose experimental group, RB1-RB3 low-dose experimental group, RC1-RC3 are blank control group, the same as below.

Figures

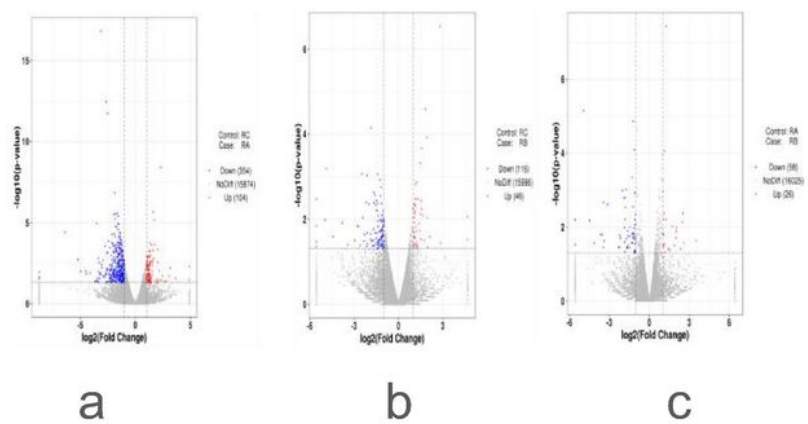
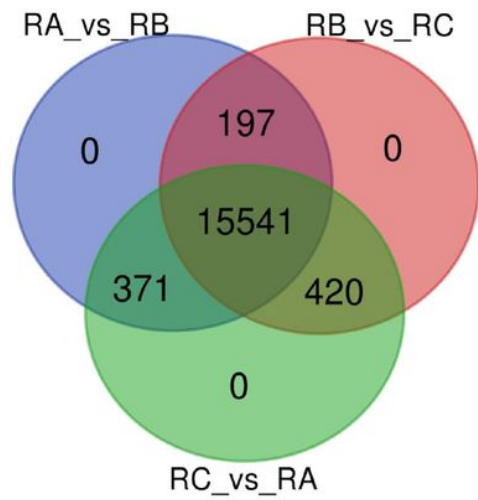


Figure 1

Volcano map of differentially expressed genes

a: (RC_vs_RA); b: (RC_vs_RB); and c: (RA_vs_RB).

a



b

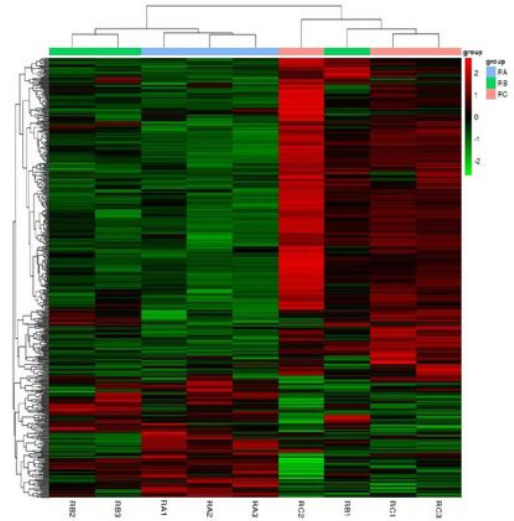


Figure 2

a Venn plot of differentially expressed genes

b Clustering heatmap of differentially expressed genes

Note: Horizontal representation of genes indicating each column of a sample; red represents high expression, and green represents low expression.

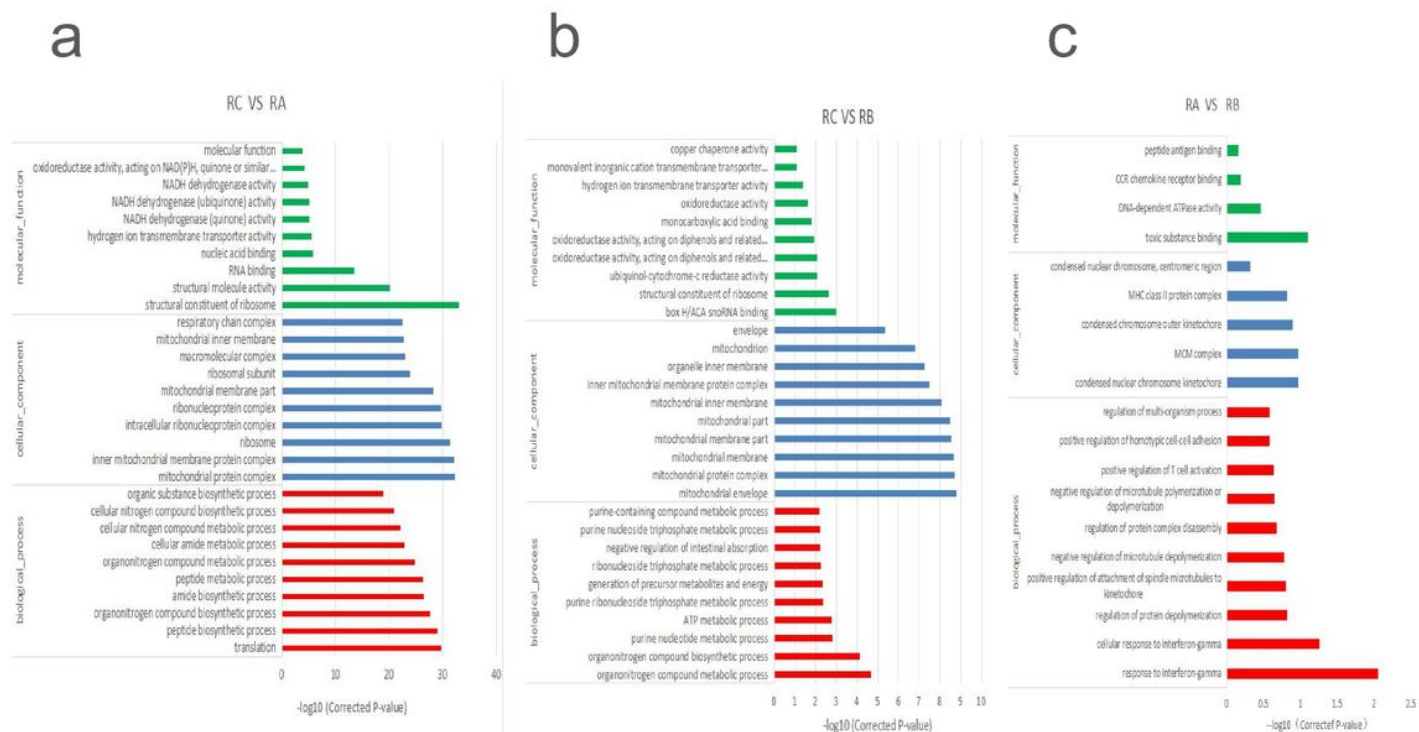


Figure 3

GO enrichment analysis of DEGs in control group and high dose group a:(RC_vs_RA); b:(RC_vs_RB); c: (RA_vs_RB).

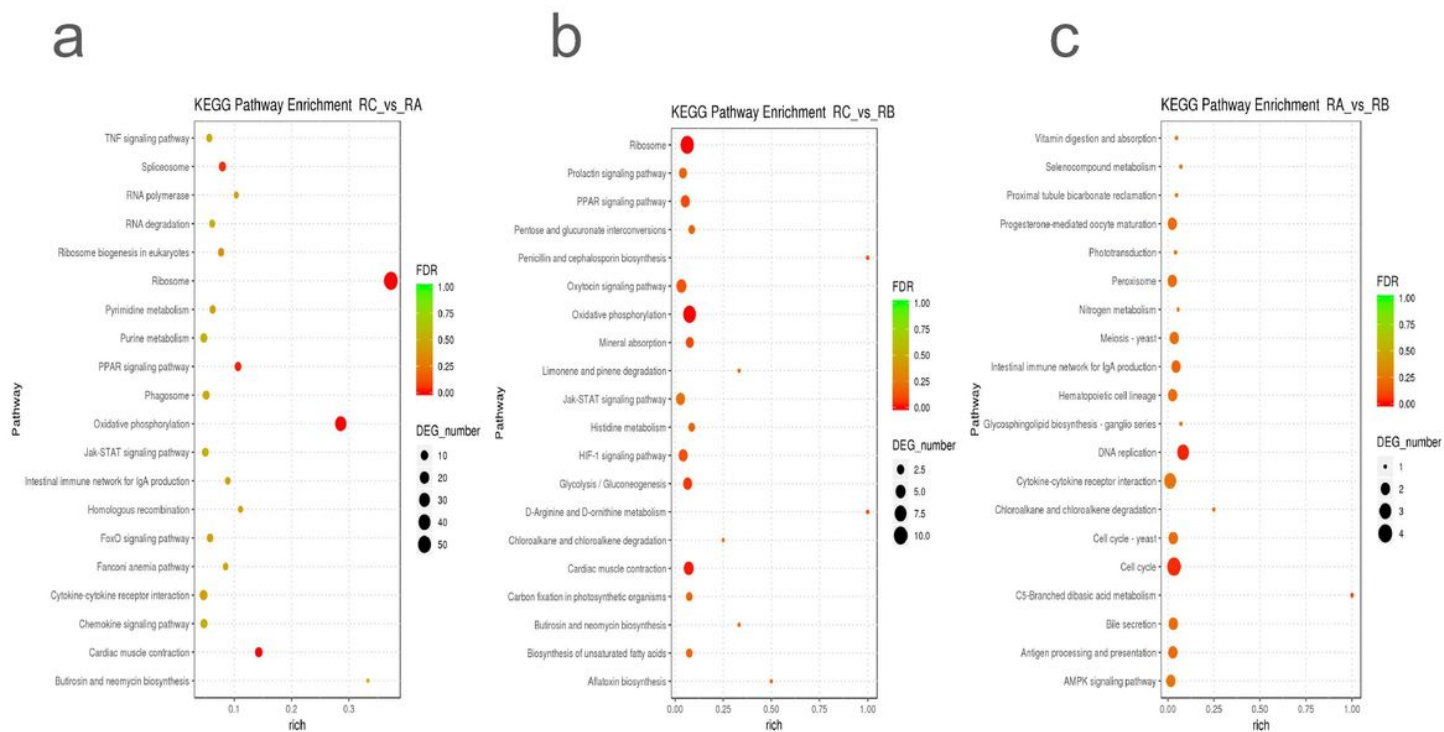
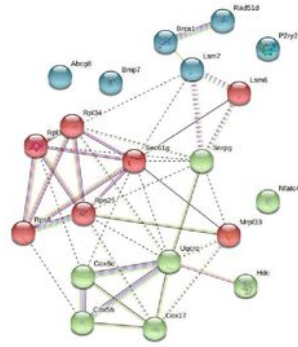


Figure 4

Figure legend not available with this version.

a



b

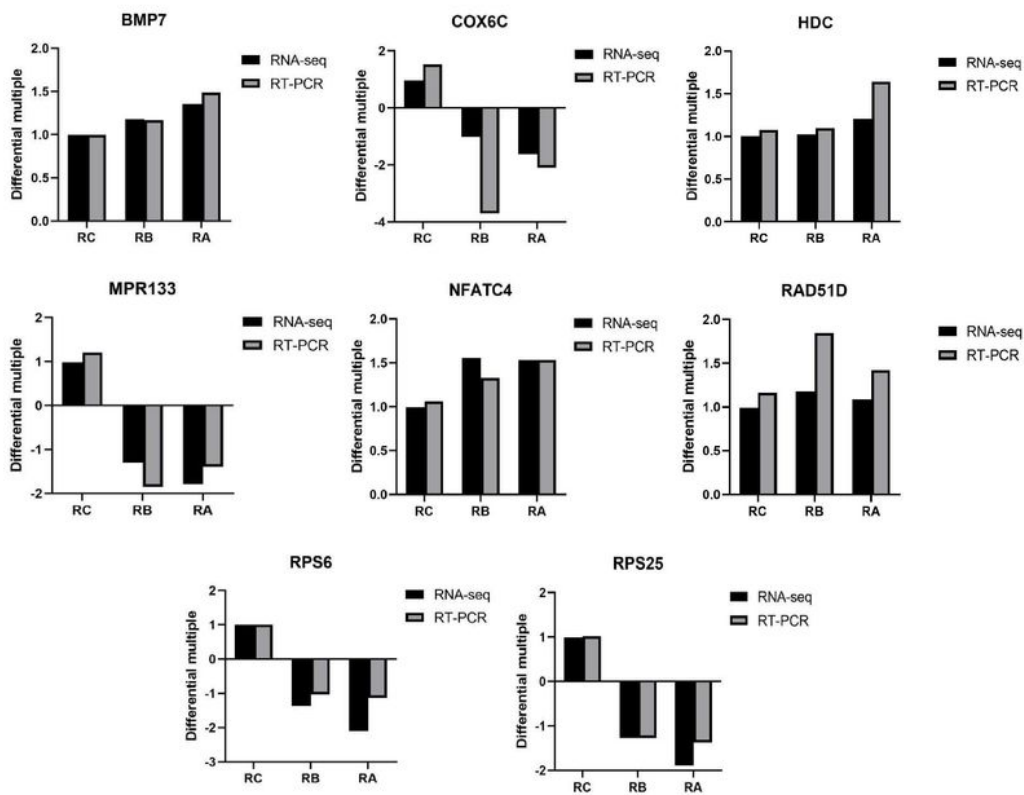


Figure 5

a Differential expressed gene interaction network analysis

b qRT-PCR validation of key genes

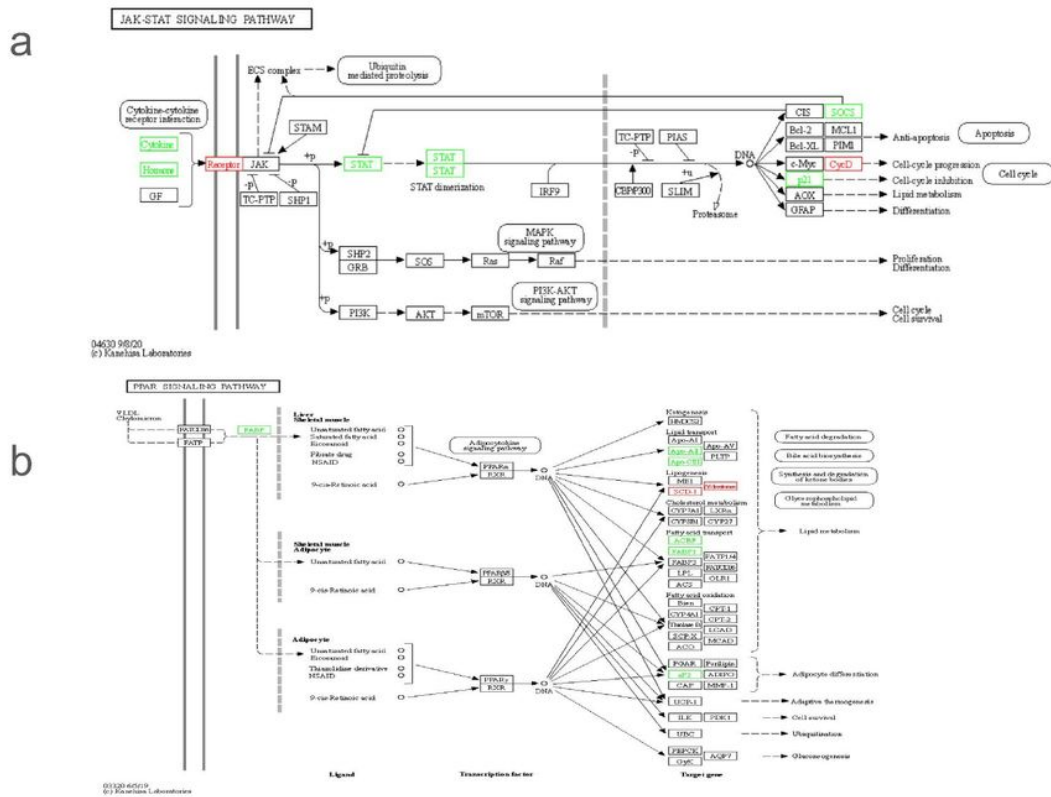


Figure 6

Combined KEGG and up-down pathway analysis

a: Jak-STAT signaling pathway; b: PPAR signaling pathway