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Ovariectomy May be Responsible for Subcutaneous Fat Deposition by Altering Hepatic Lipid Metabolism in Female Pigs

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ABSTRACT

Background: Emerging experimental and human evidence has linked decreased ovary-origin hormones and obesity and various metabolic diseases through affecting homeostasis of glucose and lipid metabolism, but the underlying mechanisms remain unclear. **Methods:** To investigate the effect removal of ovary on the growth and fat deposition of female pigs. In this work, we compared the average backfat thickness, hepatic lipid metabolism indexes and hepatic transcriptome between ovariectomized (OVX) and sham-operated (Sham) female pigs.

Result: Results showed that the OVX females had remarkably increased backfat thickness, total cholesterol and triglyceride levels in the liver, whereas serum estradiol levels and hepatic high-density lipoprotein-cholesterol levels presented significantly lower levels compared with those in the sham females. A total of 414 differentially expressed genes (DEGs) identified by transcriptome analysis between the OVX and Sham livers, of which 130 and 314 genes were up-and down-regulated in the OVX livers, respectively. Pathway analysis showed that these DEGs were mainly involved in PPAR signaling pathway, insulin signaling pathway, insulin resistance, hippo signaling pathway. Besides, twelve DEGs involved in glucose and lipid metabolism were screened out, including *PCK1*, *FDPS*, *HMGCR*, *HMGCS1*, *HMGCS2*, *PPP1R3B*, *PPP1R3C*, *ACAT2*, *SIK1*, *OGDHL*, *SOCS2* and *IGFBP1*. These results indicate that hormones generated by ovaries may play important roles in subcutaneous fat deposition by mediating hepatic glucose and lipid metabolism in female pigs.

Key words: Hepatic transcriptome, Ovariectomy, PPAR signaling pathway, Triglyceride.

INTRODUCTION

Clinical studies have shown that cessation of ovarian hormone production in menopause suffered from increased body weight gain, visceral adiposity and disorders associated with obesity-related comorbidities (Boldarine *et al*., 2020). The role of estrogens in metabolic, immune and inflammatory processes has been elaborated in both humans and rodents, although the complexity by which these effects occur is not fully understood. Previous researches in animals indicated that removal of ovaries by ovariectomy (OVX) resulted in significant changes in production performance, slaughter performance, blood biochemical indices, fat metabolism, bone development and immunity (Amorim *et al*., 2016). Elevated evidence demonstrated that OVX has an apparent effect on lipid metabolism, which favors lipogenesis but impairs fatty acid oxidation and induces a pro-inflammatory state in rats (Boldarine *et al*., 2020).

Estrogen biosynthesis mainly originates from the ovaries, which plays an important role in regulating reproductive activity, tissue metabolism and various pathophysiologic processes. All estrogen receptors are predominantly expressed in reproductive organs such as prostate, ovary, uterus, testis and breast, as well as highly expressed in the liver of humans and experimental animals (Ruggiero and Likis, 2002). In animals, OVX leaded to insulin resistance and increased susceptibility to the deleterious effects of a high-fat diet, which can be prevented by estrogen supplementation to ensure physiologic levels of this hormone (Stubbins *et al*., 2012).

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The liver is a critical organ in regulating lipid metabolism *via* secretion and excretion of bile. Accumulated studies have shown that estrogen replacement therapy greatly reduced the incidence of liver lipid deposition, suggesting that estrogen has a protective effect on hepatic lipid metabolism (Kur *et al*., 2020). So far, pigs are regarded as the most ideal organ xenograft donor of human beings because they have similar organ size, physiological metabolism and immune system (Xi *et al*., 2022). Hence, we explored the effect of OVX on hepatic lipid metabolism

of sows by comparing the hepatic gene expression by RNA sequencing in the present study.

MATERIALS AND METHODS

Sample collection

A pool of 20 Tibetan-ginger crossbred female pigs with similar age, genetic background, body weight and health were selected. Ten pigs were surgically removed ovary by bilateral ovariectomy (OVX) after induction of anesthesia (xylazine, 2.0 mg/kg bw) at postnatal 45 days of age. Remaining female pigs were treated as sham-operated controls, which underwent the entire surgery except for the removal of ovaries (Horigan *et al*., 2009). All pigs were fed with a standard diet and had free access to water until 12 h of fasting at the age of 10 months and then 5 mL of blood was collected from the jugular vein and then centrifuged at 2,600 rpm/min for 5 min after 40 min of resting and then the serum was collected. After slaughtering, the backfat thickness of the left carcass of sows at the thickest part of the shoulder the last rib and the lumbar-recommending vertebrae were measured and liver tissues were collected to perform RNA sequencing and biochemical indexes analysis. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Biochemical analysis

The levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) in the liver were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum estradiol level was analyzed with an enzyme immunoassay test kit (Ruixin Biotechnology Limited, Fujian, China) according to the protocol and manufacturer's instructions.

Total RNA extraction, library construction and sequencing

Total RNA was extracted from liver tissues (OVX, n = 4; Sham, n = 4) for RNA sequencing using a total RNA Kit (Omega, Norcross, GA, USA) according to the manufacturer's instructions. Raw data were deposited in the NCBI Sequence Read Archive database under accession number PRJNA1033387. The library preparations were sequenced on an Illumina Novaseq 6000 and 150 bp paired-end reads were generated. Low-quality reads with junctions were removed from the original sequenced sequences to obtain clean reads for subsequent analysis. Clean reads were aligned with the designated genome using HISAT2 software to obtain the positional information of the reads in the *Sus scrofa* reference genome (version: Scrofa11.1). Fragments per Kilobase Million Mapped Reads (FPKM) value of each gene then was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis of genes were then performed using DESeq2 *and* the *P*-value <0.05, |log2 (fold change)| >1 were used as the criteria for screening differentially expressed genes (DEGs). KOBAS (http://kobas.cbi.pku.edu.cn/) was used to perform the GO enrichment and KEGG pathway analysis.

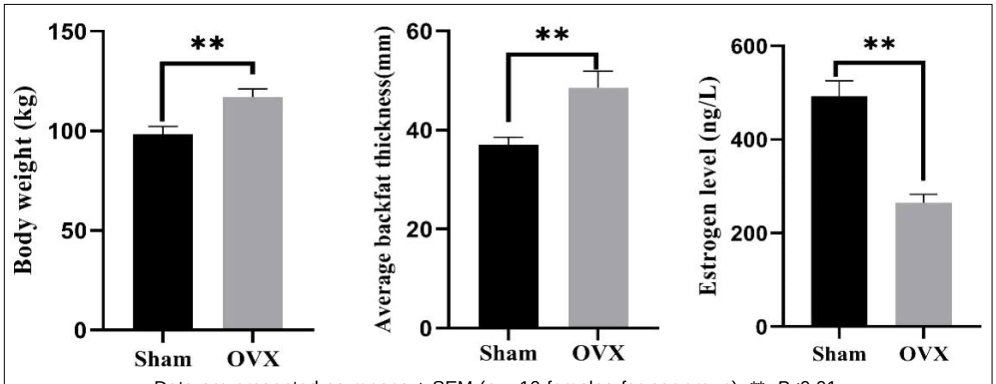
Quantitative real time polymerase chain reaction (qRT PCR) validation

Primers information was shown in Table 1. The qRT-PCR reaction was performed using SYBR Select Master Mix on the Quant Studio™3 Flex qRT-PCR System (Thermo Fisher Scientific Waltham, MA, USA). The reaction program was as follows: 50°C for 2 min UDG enzyme activation, 95°C for 2 min pre-denaturation; the PCR instrument was cycled 40 times (95°C for 15 s, 60°C for 15 s, 72°C for 1 min); and the lysis curve conditions: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method, with results run in quadruplicate.

RESULTS AND DISCUSSION

Comparison of backfat thickness and serum estradiol levels between OVX and Sham groups

We observed that the backfat thickness of female pigs in the OVX group was remarkably higher than that in the Sham group (P<0.05), while the estradiol level was significantly lower than that in the sham-operated pigs (472.49±27.45 ng/L VS. 238.71±11.30 ng/L) (P<0.05, Fig 1), which indicates that removal of ovary lead to



Data are presented as means ± SEM (n = 10 females for per group). **, P<0.01.

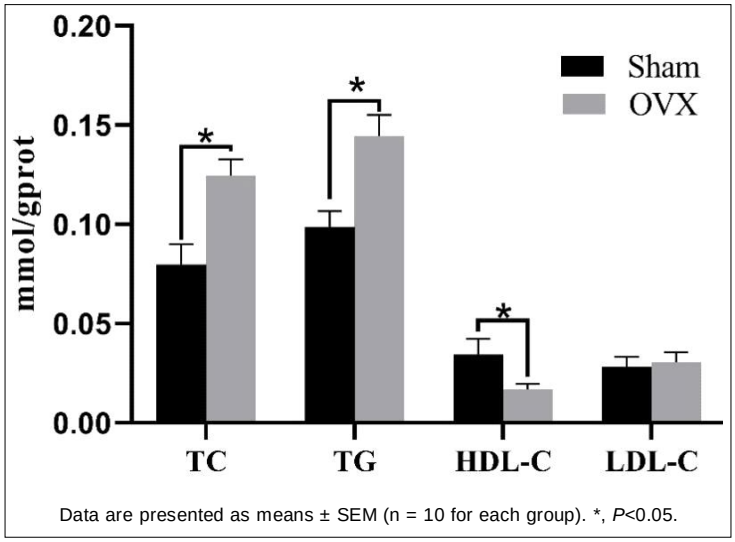
Fig 1: Differences in body weight, average backfat thickness and serum estradiol levels between the OVX and the Sham females.

subcutaneous fat accumulation in pigs. Given that the liver was essential to regulate glucose and lipid metabolism, alterations in hepatic physiological functions may be responsible for obesity of pigs.

Comparison of hepatic lipid metabolism indexes and histopathology between OVX and sham groups

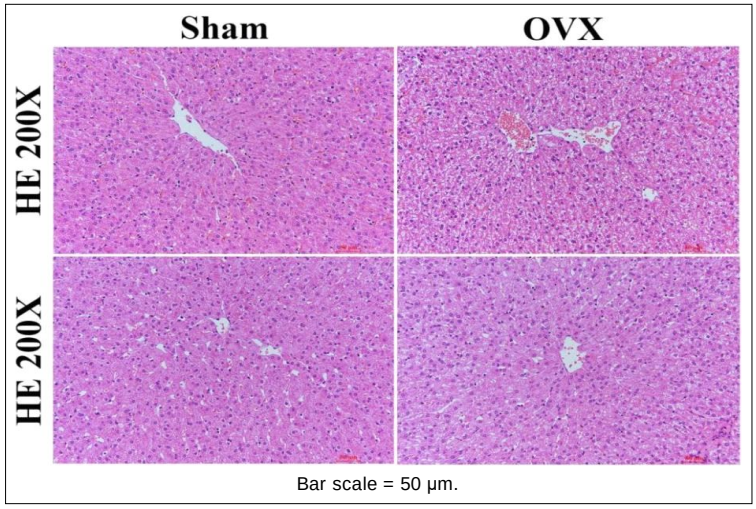
Compared with sham-operated controls, OVX pigs exhibited markedly increased levels of TC and TG and reduced levels of HDL-C (P<0.05, Fig 2). These results were consistent with previous studies in mice and rats (Zhu *et al*., 2023). High TG caused by any reason can lead to hepatic steatosis (Miller, 2000). It has been reported that the plasma TC was increased in the aging humans and rodents since their physiological function in eliminating cholesterol decreased (Lei *et al*., 2021). Several studies have demonstrated that ectopic accumulation of lipid within liver could specifically cause hepatic insulin resistance in humans and rodents (Fabbrini *et al*., 2009). A previous study found that ovariectomized mice had increased monounsaturated fatty acid levels of hepatic triglyceride in the liver (Jackson *et al*., 2011).

Histomorphologic analysis showed that OVX females had mor1e lipid drops accumulated in the livers compared to Sham pigs (Fig 3). OVX mice commonly demonstrate increased body weight owing to excessive fat accumulation, as well as increased liver lipid accumulation, leading to fatty liver (Nanashima *et al*., 2020). Hepatic cholesterol accumulation is driven by a deeply deranged cellular cholesterol homeostasis, characterized by elevated cholesterol synthesis and uptake from circulating lipoproteins and by a reduced cholesterol excretion (Musso *et al*., 2013).



Data are presented as means ± SEM (n = 10 for each group). *, P<0.05.

Fig 2: Hepatic parameters of lipid metabolism in ovariectomized pigs compared to sham-operated controls.



Bar scale = 50 μm.

Fig 3: The histomorphology of livers from ovariectomized and Sham-operated control pigs *via* H and E staining (n = 6 livers for per group).

Transcriptomic analysis of liver tissues of OVX and Sham groups

In the present study, average number of raw reads, clean reads and mapped reads in the liver of Sham and OVX groups were shown in Supplementary Table 1. By alignment analysis, 21,832 genes commonly expressed in the liver tissues of the OVX and Sham female pigs. A total of 414 genes were identified as differentially expressed genes (DEGs) between two groups under the thresholds of $P<0.05$ and |log2Foldchange| >1, of which 130 and 314 DEGs were up-regulated and down-regulated in the OVX group. Hierarchical clustering showed a very clear separation of DEGs between the OVX and Sham groups (Fig 4).

Gene ontology (GO) enrichment and KEGG pathway analysis of all DEGs were carried out between the two groups (Fig 5). GO enrichment analysis revealed that main enriched biological processes were cell-cell signaling, G protein-coupled adenosine receptor signaling pathway, cholesterol biosynthetic process. For GO cellular components, DEGs involved in the extracellular matrix, perinuclear region of cytoplasm, integral component of plasma membrane and integrin complex were most strongly represented. In the category of GO molecular function, DEGs were mainly associated with metallocarboxy peptidase activity, calcium ion binding, creatine kinase activity and protein kinase activator activity. KEGG pathway analysis showed that PPAR signaling pathway, insulin signaling pathway, hippo signaling pathway, bile secretion seemed to be the obvious core metabolic pathways. Furthermore, we screened out twelve DEGs involved in glucose and lipid metabolism, including *PCK1*, *FDPS*, *HMGCR*, *HMGCS1*, *HMGCS2*, *PPP1R3B*, *PPP1R3C*, *ACAT2*, *SIK1*, *OGDHL*, *SOCS2* and *IGFBP1* (Table 2). The qRT-PCR analysis revealed that mRNA expression levels of these genes were consistent with the results of RNA-Seq ($P<0.05$, Fig 6).

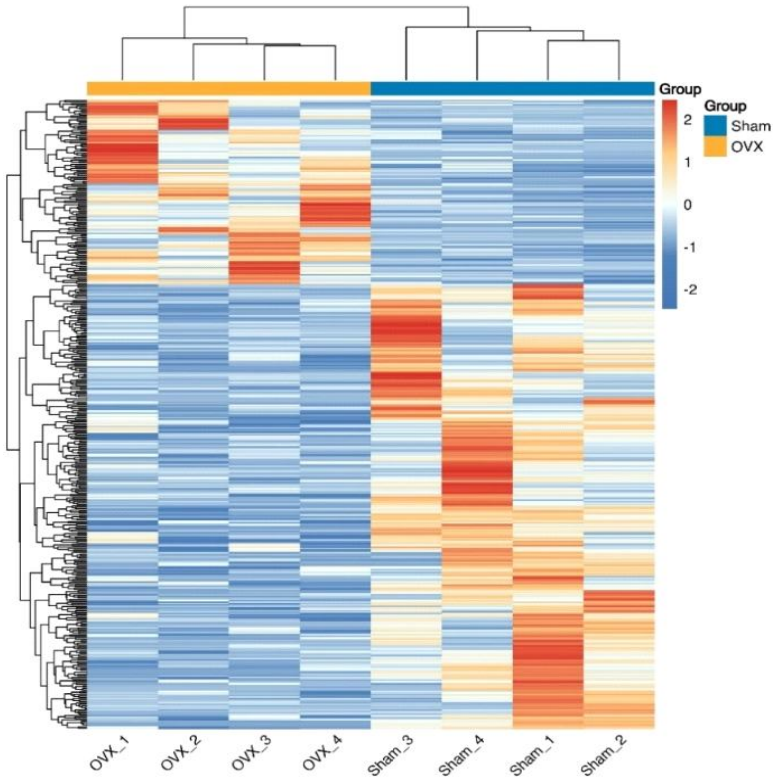
PPAR are ligand-activated transcriptional modulators with well-documented functions in hepatic, whole-body energy homeostasis, lipid and glucose metabolism *and* inflammatory responses (Berthier *et al*., 2021). A study by Hu *et al.* (2021) highlighted that hepatic lipid metabolism in the hyperlipidemia model rats could be regulated by atorvastatin ester through the PPAR signaling pathway and *HMGCR* expression. In the present study, we observed the OVX females had reduced expression levels of *PCK1* and *HMGCS2*, whereas increased levels of *HMGCR* and *HMGCS1* compared with the Sham females. Accumulating evidence indicates that *PCK1*, the rate-limiting enzyme in gluconeogenesis, regulates not only glucose homeostasis but also lipogenesis by activating sterol-regulatory

Table 1: The primers used for quantitative PCR.

Gene name	Primer sequences (5′-3′)	Length (bp)	GenBank accession no.
PCK1	F: GCAACACCATCTTCACCAA	286	NM_001123158
	R: AGAGCCTCGTACACCAGA		
HMGCS1	F: CTCCGAGAAGACACTCATCAC	187	NM_001252215
	R: GGTGGCTGTGCTTGAATGGA		
ACAT2	F: TGTCCACCGTTCCTGTCCAT	282	XM_001928345
	R: TGCCTCCTGCGACCACAAT		
HMGCS2	F: CCTCTTCAACGCTGCCAACTG	277	NM_214380
	R: CCGAAGGTAGCACTGGATGGA		
HMGCR	F: AGCAGCCGAATCCTCGCAGAT	218	NM_001122988
	R: GCATCACCTGACCTGGACTGGA		
FDPS	F: TGCTGACTGAGGAGGACATCGG	232	XM_001185131
	R: TCGTCTGCCACCAGGAAGAAGG		
PPP1R3B	F: GCAGCAAGAATGAAGCCAGTGG	295	XM_021075038
	R: ACGCAGTTCTCCAGGCAGACA		
PPP1R3C	F: CCTTCGCACAACCAAGCCAAGA	264	XM_005671280
	R: AGAGCAGTTCTCCAGGCAGACA		
OGDHL	F: CGACCAGTTCATCAGCACAG	154	XM_021072996.1
	R: GTAGGCATCCGAGTCATCATTG		
SIK1	F: TGCTGCTGCTGCTGTATG	252	XM_021071058.1
	R: AGTGCTTGTCACGGTTCTG		
SOCS2	F: CTCGGAATGCAGCTCTGTGA	193	XM_013998084.2
	R: AATGGTCTCGGTCAGTCTTACG		
IGFBP1	F: CTGCCAGCGAGAACTCTACAA	204	NM_001195105.1
	R: GTGGAGCCCAGGATCTTCTTC		
β-ACTIN	F: CCACGAGACCACCTTCAACTC	131	DQ845171
	R: TGATCTCCTTCTGCATCCTGT		

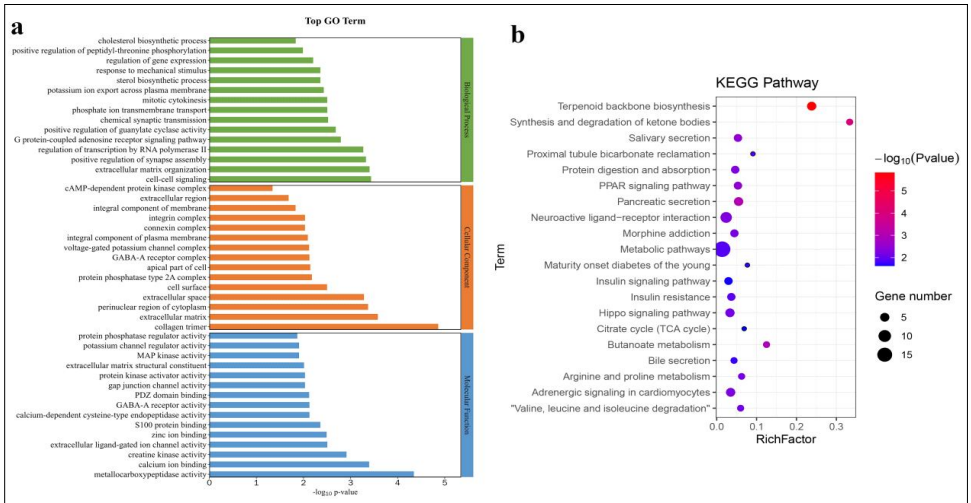
element-binding proteins (Ye *et al*., 2023). A recent study showed that *PCK1* deficiency stimulated lipogenic gene expression and lipid synthesis and activated the RhoA/PI3K/AKT pathway by increasing intracellular GTP levels, increasing secretion of platelet-derived growth factor-AA and promoting hepatic stellate cell activation (Ye *et al*., 2023).

The steady state of the cholesterol metabolism depends on a complex network involving cholesterol uptake, synthesis, transport and excretion (Musso *et al*., 2013).



Red color indicates relatively highly expressed genes and blue color indicates relatively low expressed genes.

Fig 4: Heatmap for clustering analysis of DEGs.



(a) GO enrichment analysis. (b) KEGG pathway analysis of DEGs.

Fig 5: GO enrichment and KEGG pathway analysis of DEGs in the liver tissues between OVX and sham groups.

Many clinical and basic research have confirmed that lipid metabolism is bound up with cholesterol synthesis (Luo *et al*., 2023). Both *HMGCS* and *HMGCR* are the two key rate-limiting enzymes in cholesterol synthesis (Hu *et al*., 2021). A study conducted on OVX mice model suggested that the expression level of *HMGCR* related with cholesterol metabolism also significantly increased in the liver of ovariectomized mice compared with sham mice (Lei *et al*., 2021). Besides, several noncoding RNA has been found to regulating hepatic cholesterol and lipid metabolism by targeting *HMGCR* (Sun *et al*., 2015). Additionally, in cholesterol metabolism, ACAT2 promotes the secretion of cholesteryl ester-enriched very low-density lipoproteins by the liver and lacking hepatic ACAT2 can prevent dietary cholesterol-driven hepatic steatosis in mice (Alger *et al*., 2010). Hence, OVX females had elevated *ACAT2* expression may be related to increase of hapetic TC levels.

In the liver, two major subunits, protein phosphatase 1 regulatory subunit 3B(*PPP1R3B*) and regulatory subunit 3C (*PPP1R3C*) are expressed at approximately equivalent levels and together facilitate the storage of hepatic glycogen. It has been confirmed that overexpression of *PPP1R3B* lead to increase of liver glycogen in human cell lines and in mice (Agius, 2015). Furthermore, López-Soldado *et al*. (2015) found that *PPP1R3C* transgenic mice increased hepatic glycogen and improved glucose and insulin sensitivity. A survey proved that hepatic *PPP1R3C* mRNA expression in diabetic mice was increased compared with that in control mice, suggesting that the abnormally high expression of *PPP1R3C* in livers may be one of the main causes of hyperglycemia in diabetes (Ji *et al*., 2019). Therefore, the expression levels of *PPP1R3B* and *PPP1R3C* in the livers of OVX females were upregulated in this study, which may imply ovariectomy affects liver glucose metabolism.

Salt-inducible kinase 1 (*SIK1*) belongs to the serine-threonine kinase family *and* also is an AMPK-related protein kinase (Darling and Cohen, 2021). A recent study reported that the overexpression of *SIK1* in the liver of the rats could downregulate the expression of gluconeogenic genes (Song *et al*., 2019). In primary mouse hepatocytes, forced *SIK1* expression not only promoted gluconeogenesis but also suppressed lipogenesis (Wang *et al*., 2020). Consistent with this, data from Zhang *et al*. (2017) demonstrate that *SIK1* expression is inversely correlated with the expression of lipogenic molecules and lipid biosynthesis. In the present study, downregulation of *SIK1* in the OVX livers may facilitate gluconeogenic process.

Oxoglutarate dehydrogenase-like (*OGDHL)* participates in regulating the degradation of glucose and glutamate (Bunik and Degtyarev, 2008). A study conducted on hepatocellular carcinoma suggested that silencing of *OGDHL* result in decrease of lipogenesis (Dai *et al*., 2020),

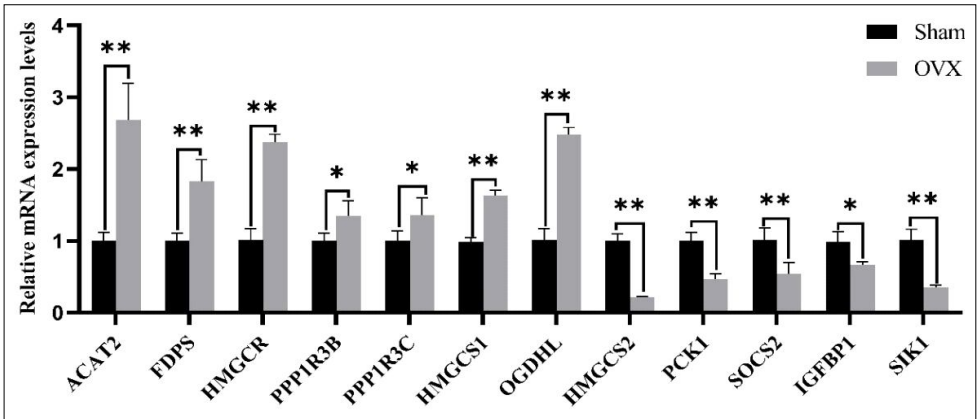


Fig 6: Validation of DEGs involved in lipid metabolism in liver tissues between OVX and Sham groups by qRT-PCR.

Supplementary Table 1: Basic information on transcriptome sequencing of each liver tissue.

Sample	Raw reads	Clean reads	Totalmapped	Multiple mapped	Unique mapped
Sham_1	50664230	50660714	47279520 (93.33%)	2230223 (4.40%)	45049297 (88.92%)
Sham_2	40832258	40830270	38088516 (93.28%)	1717789 (4.21%)	36370727 (89.08%)
Sham_3	47342878	47341304	56362015 (93.85%)	2654132 (4.42%)	53707883 (89.43%)
Sham_4	47860746	47857772	43295809 (92.52%)	2059841 (4.40%)	41235968 (88.12%)
OVX_1	60061474	60057904	43729604 (92.37%)	2046771 (4.32%)	41682833 (88.05%)
OVX_2	46798892	46796476	44808967 (93.63%)	1879614 (3.93%)	42929353 (89.70%)
OVX_3	48502148	48498872	45001447 (92.79%)	3488869 (7.19%)	41512578 (85.59%)
OVX_4	47306980	47305018	44519945 (94.11%)	2168965 (4.59%)	42350980 (89.53%)

Table 2: List of differentially expressed genes involved in glucose and lipid metabolism between the OVX and sham livers.

Gene symbol	Accession	log2FC	P-value	Gene description	KEGG pathway
PCK1	ENSSSCG00000007507	-1.59	0.0045	Phosphoenolpyruvate carboxykinase 1	PPAR signaling pathway; Insulin resistance; Proximal tubule bicarbonate reclamation; Insulin signaling pathway; Citrate cycle (TCA cycle); Glucagon signaling pathway; Pyruvate metabolism
FDPS	ENSSSCG00000006512	1.16	0.0000	farnesyl diphosphate synthase	Terpenoid backbone biosynthesis
HMGCR	ENSSSCG00000014080	1.15	0.0377	3-hydroxy-3-methylglutaryl-CoA reductase	Terpenoid backbone biosynthesis; Bile secretion
HMGCS1	ENSSSCG00000016872	1.30	0.0068	3-hydroxy-3-methylglutaryl-CoA synthase 1	PPAR signaling pathway; Terpenoid backbone biosynthesis; Synthesis and degradation of ketone bodies; Butanoate metabolism; Valine, leucine and isoleucine degradation
HMGCS2	ENSSSCG00000036808	-1.74	0.0345	3-hydroxy-3-methylglutaryl-CoA synthase 2	PPAR signaling pathway; Terpenoid backbone biosynthesis; Synthesis and degradation of ketone bodies; Butanoate metabolism; Valine, leucine and isoleucine degradation
PPP1R3B	ENSSSCG00000022797	1.55	0.0100	protein phosphatase 1 regulatory subunit 3B	Insulin resistance; Insulin signaling pathway
PPP1R3C	ENSSSCG00000010464	1.24	0.0042	protein phosphatase 1 regulatory subunit 3C	Insulin resistance; Insulin signaling pathway
ACAT2	ENSSSCG00000004049	1.04	0.0000	acetyl-CoA acetyltransferase 2	Terpenoid backbone biosynthesis; Synthesis and degradation of ketone bodies; Butanoate metabolism; Valine, leucine and isoleucine degradation; Pyruvate metabolism
SIK1	ENSSSCG00000035037	-1.77	0.0028	salt inducible kinase 1	Glucagon signaling pathway
OGDHL	ENSSSCG00000010394	1.30	0.0245	oxoglutarate dehydrogenase L	Citrate cycle (TCA cycle)
SOCS2	ENSSSCG00000053891	-1.22	0.0121	suppressor of cytokine signaling 2	Insulin signaling pathway
IGFBP1	ENSSSCG00000016728	-2.10	0.0039	insulin like growth factor binding protein 1	-

so upregulation of *OGDHL* may be associated with elevated triglyceride levels.

Insulin-like growth factor binding protein 1 (*IGFBP1*), a binding protein with a high affinity to insulin-like growth factors, mainly produced by hepatocytes and is proved to be associated with glucose regulation and insulin resistance. *IGFBP1* treatment significantly ameliorated hepatic steatosis by interacting with *ITGB1* and suppressed inflammation by inhibiting NF-κB and ERK signaling pathways (Pan *et al*., 2021). Previous research has indicated that reduced levels of serum IGFBP1 are commonly observed in obesity, hyperinsulinemia and nonalcoholic fatty liver disease (NAFLD) (Graffigna *et al*., 2009). Furthermore, as one of the suppressors of cytokine signaling (*SOCS*) family, it has been clarified that *SOCS2* participated in hepatic steatosis and NAFLD (Yuan *et al*., 2016). Recent research published demonstrated that *SOCS2* plays a role in inhibiting inflammation and apoptosis in macrophages during nonalcoholic steatohepatitis through the NF-κB and inflammasome signaling pathways (Li *et al*., 2021). Therefore, decreased expression levels of *IGFBP1* and *SOCS2* in the OVX livers may increase the risk of hepatic steatosis.

CONCLUSION

In conclusion, ovariectomy resulted in increase of backfat thickness and lipid accumulation in hepatocytes. Transcriptomic analysis suggested that ovariectomy altered expression of important genes and pathways involved in glucose and lipid metabolism to affect hepatic lipid homeostasis, which may be associated with obesity of OVX pigs. The present study provides valuable information for understanding the ovary-origin hormones roles in hepatic lipid metabolism and fat deposition of female pigs.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Institutional Animal Care and Use Committee of Guizhou University, Guiyang, PR China (EAE-GZU-2021-T053).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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