



Article

FOXA3 Alleviates Lipid Deposition in Primary Bovine Hepatocytes by Inhibiting SREBP1 and Cell Proliferation

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Simple Summary

During the periparturient period, dairy cows often experience negative energy balance, leading to elevated levels of non-esterified fatty acids (NEFA) in their blood. This condition predisposes them to fatty liver disease and ketosis. *FOXA3*, a crucial transcription factor involved in regulating liver metabolism, has an unclear role in the pathogenesis of fatty liver in dairy cows. This study aimed to elucidate the mechanism by which *FOXA3* regulates hepatic lipid metabolism. We analyzed liver samples from dairy cows diagnosed with fatty liver, developed a lipid accumulation model using primary hepatocytes treated with NEFA, and employed *FOXA3* overexpression technology. Our findings revealed a significant reduction in *FOXA3* expression in the livers of dairy cows with fatty liver. Furthermore, NEFA were found to downregulate *FOXA3* expression and promote lipid accumulation. In contrast, *FOXA3* overexpression mitigated lipid accumulation by inhibiting lipogenesis-related genes, such as *SREBP1*, and influencing cell cycle pathways. This study elucidates the protective role of *FOXA3* in the development of fatty liver in dairy cows, indicating that enhancing *FOXA3* expression could be a novel strategy for the prevention and treatment of fatty liver and ketosis in dairy cows, with significant economic value.

Abstract

During the periparturient period, negative energy balance (NEB) in dairy cows leads to increased concentrations of non-esterified fatty acids (NEFA) in the blood, which can induce fatty liver disease and ketosis. Forkhead box protein A3 (*FOXA3*) is a key transcription factor that regulates liver metabolism; however, its specific role in the pathogenesis of fatty liver in dairy cows remains unclear. This study aimed to investigate the mechanism by which *FOXA3* regulates hepatic lipid metabolism. We collected liver samples from dairy cows with fatty liver ($n = 10$) and from healthy cows ($n = 10$). Bovine primary hepatocytes were isolated from the liver tissue of calves ($n = 5$), followed by NEFA treatment, and we utilized *FOXA3* overexpression, immunofluorescence, and RNA sequencing (RNA-seq) to conduct our analysis. Our results demonstrated that *FOXA3* expression in the livers of cows with fatty liver was significantly lower than in healthy cows. NEFA treatment resulted in the downregulation of *FOXA3* protein levels in hepatocytes, promoting triacylglycerol (TAG) accumulation and the expression of lipogenesis-related genes. Conversely, *FOXA3* overexpression mitigated NEFA-induced lipid accumulation, inhibited the expression of



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lipogenesis-related genes and proteins—particularly SREBP1—and affected cell proliferation, and the intracellular localization of *FOXA3* and *SREBP1*. RNA-seq analysis suggested that *FOXA3* may influence hepatic lipogenesis through pathways such as PI3K-Akt and the cell cycle. In summary, *FOXA3* mitigates NEFA-induced hepatic lipid accumulation through a dual mechanism: regulating *SREBP1* expression and inhibiting cellular proliferation. These findings highlight *FOXA3*'s potential as a novel target for the prevention and treatment of fatty liver disease in dairy cows.

Keywords: fatty liver; *FOXA3*; Ketosis; lipid accumulation; primary bovine hepatocytes

1. Introduction

Diseases that occur around the time of calving in dairy cows significantly impact both the yield and quality of milk. During the periparturient period, particularly among high-producing dairy cows, NEB often develops due to increased energy demands from lactation and decreased dry matter intake [1]. In a prolonged state of NEB, cows initiate an intensive fat mobilization process, leading to elevated levels of NEFA in the bloodstream. Excessive NEFA are then transported to the liver, where they are metabolized through both re-esterification and incomplete β -oxidation pathways. This process ultimately results in excessive production of triacylglycerol (TAG) in the liver and the simultaneous generation of substantial amounts of ketone bodies [2–4], which can lead to fatty liver disease or ketosis [5,6]. Both conditions are the most common hepatic metabolic diseases observed in periparturient dairy cows and are closely linked in terms of etiology and pathogenesis, often occurring simultaneously or acting as predisposing factors for one another [7,8]. Fatty liver can be classified based on TAG content as normal ($\text{TAG} < 1\%$), mild ($1\% \leq \text{TAG} < 5\%$), moderate ($5\% \leq \text{TAG} < 10\%$), or severe ($\text{TAG} \geq 10\%$) [6,9]. Ketosis and fatty liver can also lead to other diseases, such as mastitis and abomasal displacement, which not only reduce milk production but also severely impair the reproductive performance of dairy cows, resulting in significant economic losses for the dairy industry [6]. Therefore, it is crucial to investigate the molecular biology underlying the pathogenesis of fatty liver in dairy cows to identify potential targets for its prevention and treatment.

Forkhead box protein A3 (*FOXA3*) is a member of the winged helix transcription factor family. While it is primarily localized in the cell nucleus, its expression patterns vary significantly among different cell types [10,11]. *FOXA3* is present in various organs, including the liver, where it plays a critical role in glucose and lipoprotein metabolism, as well as liver growth [12–15]. Research in mice has demonstrated that increased *FOXA3* expression reduces TAG accumulation and alleviates adiposity as well as chronic hepatic disorders [16]. Conversely, deletion of the *FOXA3* gene in mice impairs hepatocyte proliferation and exacerbates liver damage, while enhanced expression of *FOXA3* promotes hepatic regeneration and alleviates hepatocyte injury [16]. Furthermore, in human patients, overexpression of *FOXA3* inhibits hepatocellular carcinoma (HCC) growth and increases the sensitivity of HCC to anticancer therapies [17]. These findings suggest that *FOXA3* significantly contributes to the lipolysis, enhances liver metabolism, and improves developmental functions in the livers of non-ruminant species, positioning it as a crucial target for alleviating hepatic lipid accumulation and enhancing liver function. However, there are currently no reported studies on the role of *FOXA3* in the livers of ruminant animals, such as cows.

SREBP1 is a key transcription factor regulating lipid and glucose homeostasis [18,19]. Its activation promotes lipogenesis and the development of fatty liver [20,21], while its

inhibition ameliorates diet-induced hepatic steatosis [22]. Under low-sterol conditions, the *SREBP1* precursor is cleaved and translocates to the nucleus, where it activates *ACCα*, *FASN*, and other lipogenic genes [18,23–26]. *FOXA3* downregulates *SREBP1* and *ACCα* expression, thereby improving hepatic steatosis in mice via the XBP1s-*FOXA3*-PER1/*SREBP1c* axis [13,27]. Based on these findings, we hypothesize that *FOXA3* attenuates *SREBP1*-mediated lipid synthesis through as yet unidentified mechanisms.

Recent studies have established a strong link between proliferation/differentiation pathways and metabolism: the PI3K/AKT/mTOR pathway promotes anabolism and cell proliferation, whereas the LKB1/AMPK pathway inhibits anabolism and suppresses proliferation [28–30]. In mice, *FOXA3* reactivates the cell cycle in quiescent hepatocytes, facilitates liver regeneration and adipocyte differentiation, and its deletion impairs hepatocyte proliferation and exacerbates liver injury [16,31,32]. These findings suggest that *FOXA3* coordinates metabolism and proliferation; however, its role in bovine primary hepatocytes remains unclear. Therefore, we hypothesize that *FOXA3* modulates lipid metabolism by regulating proliferative activity.

In this study, we hypothesize that *FOXA3* can alleviate NEFA-induced lipid accumulation and may do so through mechanisms involving *SREBP1* and the cell cycle. Therefore, this study aims to investigate the expression status of *FOXA3* in cows with fatty liver and in bovine hepatocytes following NEFA treatment, as well as to confirm its function and mechanisms in lipid accumulation.

2. Materials and Methods

2.1. Animals and Sample Collection

The Animal Welfare Ethics Committee of Jilin University approved this trial (SY202401011), which was conducted in strict accordance with applicable regulations. The dairy cows used in this experiment were selected from a large-scale intensive farm in Changchun, where all animals were uniformly fed a total mixed ration. The cows had a similar number of lactations (median = 3, range = 2–4) and days in milk (median = 6, range = 3–9 days). Initially, a preliminary screening was performed on a group of Holstein cows with similar lactation frequencies and days in milk to conduct a comprehensive routine health examination. Following this, liver tissue samples were collected according to the methods outlined in previous research [33]. Based on liver TAG content, the cows were classified as healthy (TAG < 1%, g/g of wet weight) or severe fatty liver (TAG ≥ 10%). From this classification, 10 healthy cows and 10 cows with fatty liver were selected for further study. The basic physiological parameters of the cows are detailed in earlier research [34]. Liver samples were fixed in 4% paraformaldehyde or preserved by freezing in liquid nitrogen.

2.2. Isolation and Culture of Primary Bovine Hepatocytes

A total of 5 female Holstein calves, each one day old and weighing between 30 and 40 kg, were used in the present study. Our method for isolating primary hepatocytes employs a modified technique, with all reagents and procedures referenced from Zhu's article [35]. Initially, five one-day-old female calves were selected for anesthesia and subsequently received an intravenous injection of heparin sodium. The caudate liver lobe was excised and perfused using Solution A and Solution B, followed by digestion of the tissue with a 0.02% collagenase IV solution. The digestion was terminated by adding 10% fetal bovine serum, after which the tissue was minced and filtered. The cell suspension was washed twice with RPMI-1640 medium (Hyclone Laboratories, Logan, UT, USA), then resuspended and centrifuged using 25% Percoll for cell purification. Cells with a viability greater than 90%, as determined by trypan blue staining, were considered qualified for

experiments. Finally, the cells were plated into six-well plates. After 4 h of adherence, the medium was changed to growth medium, which was subsequently renewed every 24 h.

2.3. Preparation of NEFA

In this study, the preparation method for NEFA was adapted from previous research [36]. The specific steps are as follows: a 0.1 M KOH solution was used to dissolve the fatty acids, which were then thoroughly mixed at 60 °C. The pH was subsequently adjusted to 7.4 using 1 M hydrochloric acid. The resulting NEFA stock solution had a concentration of 52.7 mM and included oleic acid, palmitic acid, linoleic acid, palmitoleic acid, and stearic acid, all sourced from Sigma-Aldrich Co., Ltd. (Steinheim, Germany). After preparation, the NEFA stock solution was aliquoted and stored at −20 °C for future use. Prior to application, the solution was heated in a 70 °C water bath until clear and then combined with culture medium containing 2% fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich) for hepatocyte treatment.

2.4. Cell Processing

To evaluate the effects of NEFA on the expression of *FOXA3* and lipogenesis, and to determine the optimal concentration for in vitro culture, bovine primary hepatocytes were initially cultured in RPMI-1640 basic medium for 12 h. Subsequently, the medium was replaced with RPMI-1640 basic medium supplemented with 2% BSA, and the cells underwent a 12 h treatment with a gradient of NEFA concentrations (0, 0.6, 1.2 and 2.4 mM).

For *FOXA3* overexpression studies, hepatocytes were treated with adenoviruses designed by Hanheng Biotechnology Co., Ltd. (Shanghai, China). based on the coding sequence of bovine *FOXA3* (NM_001033119.2). Specifically, the bovine empty adenovirus vector (Ad-GFP) or the bovine *FOXA3* overexpression adenovirus (Ad-*FOXA3*) was initially infected with half the volume of RPMI-1640 alkaline medium for 4 h. After this period, the remaining half of the culture medium was added to continue the infection. Following 8 h of infection, the cells were transferred to growth medium for an additional 36 h. The viral titers of Ad-GFP and Ad-*FOXA3* used in this study were 3.16×10^{10} PFU/mL and 2.51×10^{10} PFU/mL, respectively.

In the *FOXA3* silencing experiment, hepatocytes were transfected with either small interfering RNA (siRNA) negative control (si-Control) or siRNA targeting *FOXA3* (si-*FOXA3*) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM I Reduced Serum Medium (Gibco, NY, USA) for 6 h. The medium was then replaced with growth medium, and the cells were cultured for an additional 42 h. The siRNA sequences were designed by GenePharma Co., Ltd. (Shanghai, China). and are listed in Supplementary Table S1.

After transfecting with Ad-*FOXA3* or si-*FOXA3* for 48 h, the medium was replaced with RPMI-1640 basic medium supplemented with 2% BSA. The cells were then exposed to 1.2 mM NEFA for 12 h.

2.5. TAG Content Determination

According to the instructions provided by the TAG Assay Kit (Applygen, Beijing, China), lysis buffer was initially added to the hepatocytes in six-well plates to facilitate cell lysis. A cell scraper was then employed to transfer the lysed cells into centrifuge tubes. Following this, 10 µL of the sample supernatant was mixed with 190 µL of the chromogenic solution and incubated at 37 °C for 15 min. The absorbance of the resulting mixture was subsequently measured using a spectrophotometer. Finally, the protein concentration was determined using a BCA Protein Assay Kit (Proteintech, Wuhan, China) in accordance with the kit's instructions. The TAG values were normalized to the protein concentration.

2.6. Oil Red O Staining

Following the manufacturer's instructions, Oil Red O solution was prepared by dissolving 0.5 g of Oil Red O powder (Sigma-Aldrich) in 100 mL of isopropanol. This solution was then kept in a dark environment at 65 °C for 48 h and subsequently diluted with double-distilled water to achieve a final concentration of 60%. All subsequent staining procedures were conducted at room temperature and in the dark. First, the cells in each well were washed three times with phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde for 10 min. The hepatocytes were then incubated in 60% isopropanol for 30 s and stained with the Oil Red O working solution for 30 min. Afterward, the hepatocytes were washed again with 60% isopropanol for 30 s and counterstained with hematoxylin. Finally, the hepatocytes were observed, and images were captured at magnifications of 40× and 20×.

2.7. Real-Time Quantitative PCR Analysis

Total RNA was extracted from liver tissues and hepatocytes using RNAiso Plus reagent (Takara, Kusatsu, Shiga, Japan). The OD₂₆₀/OD₂₈₀ ratio ranged from 1.91 to 1.98, and the 28S/18S ratio was approximately 2, indicating high purity and integrity of the RNA, which is suitable for subsequent experiments. Following the reverse transcription of 1 µg of RNA, quantitative PCR (qPCR) was conducted using SYBR Green on an ABI 7500 (Applied Biosystems, Foster City, CA, USA), adhering to the reaction protocol outlined in the instruction manual. β-actin served as the internal reference, and the results were analyzed using the $2^{-\Delta\Delta CT}$ method. The corresponding primer sequences are provided in Supplementary Table S2.

2.8. Protein Preparation and Western Blotting

Cellular proteins were extracted using a commercial kit (Mei5 Biotechnology, Beijing, China) and quantified with a BCA assay (Applygen). Protein samples (25 µg per lane) were resolved on 10% SDS-PAGE gels and electrotransferred to PVDF membranes. Following a 2 h blocking step with 5% BSA in TBST at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies against GAPDH (Proteintech), FOXA3 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), SREBP-1 (Novus Biologicals LLC, Centennial, CO, USA), and ACCα (Proteintech). After washing with TBST, the membranes were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Proteintech) for 45 min at room temperature. Signal detection was performed using an ultrasensitive ECL substrate (Meilunbio, Dalian, China), and band intensities were quantified with ImageJ software (version 1.46), normalizing to GAPDH.

2.9. Immunofluorescence Staining

Hepatocytes cultured in laser confocal dishes (Sorfa Life Science, Zhejiang, China) were fixed with 4% paraformaldehyde (Solarbio, Beijing, China) and subsequently permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). The cells were then incubated overnight at 4 °C with primary antibodies against FOXA3 (Santa Cruz) and SREBP1 (Novus). Following this, the cells were incubated with FITC-conjugated goat anti-rabbit antibody (Proteintech) for 45 min at room temperature. Finally, DAPI (Sigma-Aldrich) was used to counterstain the cell nuclei. Images were captured using an Olympus Fluoview FV1200 confocal microscope (Olympus Corporation, Tokyo, Japan).

2.10. Cell Cycle Assay

The cell cycle assay kit (Beyotime, Shanghai, China) was utilized in this experiment. Briefly, the cells were washed with PBS, fixed in 70% cold ethanol for 12 h, and subse-

quently stained with propidium iodide. For specific experimental conditions, including temperature, please refer to the kit's instruction manual. Red fluorescence was detected using a flow cytometer, and light scattering was also measured. The data were analyzed using FlowJo software version 10.8.1 (BD Biosciences, Franklin Lakes, NJ, USA).

2.11. Liver Transcriptome Sequencing

Total RNA from Ad-FOXA3 ($n = 2$) and Ad-GFP ($n = 2$) was extracted using the Trizol-chloroform method (Takara), and its concentration, purity and integrity were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The sequencing library was constructed in strict accordance with the standard protocol: mRNA with poly(A) tails was enriched using Oligo(dT)-coated magnetic beads. Following fragmentation, the mRNA was reverse-transcribed into double-stranded cDNA, which was then subjected to end repair, A-tailing, and adapter ligation, culminating in final library production through PCR amplification. After passing quality control, the library underwent PE150 sequencing on the Illumina NovaSeq 6000 platform, provided by LC-Bio Technologies Co., Ltd. (Hangzhou, Zhejiang, China).

2.12. Statistical Analysis

All data were analyzed using SPSS version 26.0 and GraphPad Prism version 10.0, with normality and homoscedasticity tests conducted prior to analysis. Comparisons between two groups were performed using an independent samples *t*-test, while comparisons among multiple groups were conducted using one-way ANOVA followed by Bonferroni correction. All experiments were repeated three times or more. Data are presented as mean $\pm$ standard deviation. A *p*-value of <0.05 was considered statistically significant, while a *p*-value of <0.01 was regarded as extremely significant.

3. Results

3.1. Hepatic FOXA3 Is Lowly Expressed Among Dairy Cows with Fatty Liver

Compared to healthy cows, liver FOXA3 expression was significantly lower in cows suffering from fatty liver disease (Figure 1A,B). Additionally, the FPKM values from RNA-seq indicated that the FOXA3 mRNA expression in cows with severe NEB was lower than that in cows with mild NEB. These findings suggest a reduced expression of FOXA3 in the livers of cows affected by both fatty liver and severe NEB (Figure 1C).

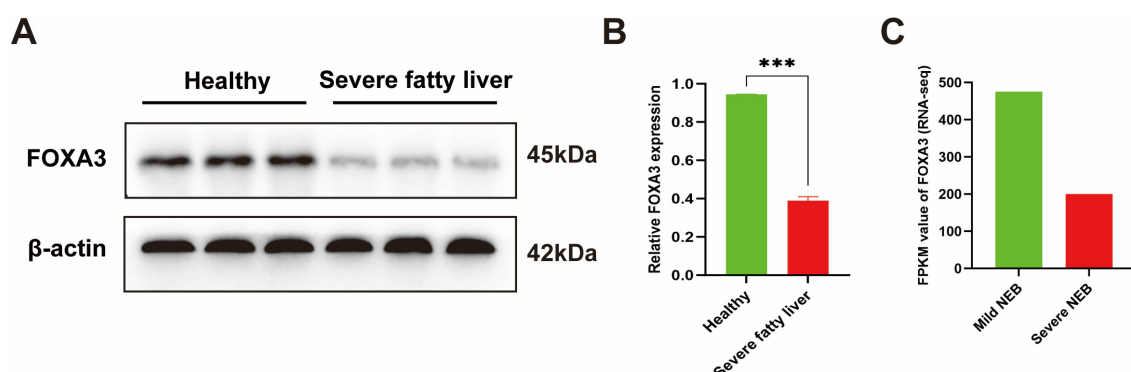


Figure 1. Hepatic FOXA3 is lowly expressed among dairy cows with fatty liver. (A,B) Protein expression of FOXA3 in the liver of healthy cows and cows with fatty liver, and quantitative analysis of grayscale values. (C) FOXA3 mRNA expression in the liver of cows with mild NEB and cows with severe NEB; Data from GSE37544. Each experiment was repeated three times. Data were analyzed using an independent sample *t*-test followed by Bonferroni correction, and the results are expressed as mean $\pm$ SEM. *** $p < 0.001$.

3.2. FOXA3 Alleviates NEFA-Induced Lipid Accumulation

Compared to the control group, treatment of calf primary hepatocytes with 0, 0.6, 1.2 and 2.4 mM NEFA for 12 h resulted in a significant decrease in the protein abundance of FOXA3 (Figure 2A,B). Calf primary hepatocytes were treated with Ad-FOXA3, 1.2 mM NEFA, or a combination of both for 24 h. Oil Red O staining revealed that NEFA increased both the size and number of lipid droplets, leading to significant lipid accumulation within the cells. However, overexpression of FOXA3 effectively mitigated this lipid accumulation (Figure 2C,D). Additionally, NEFA significantly increased the TAG content in bovine hepatocytes, while FOXA3 overexpression markedly reduced TAG levels (Figure 2E). Furthermore, when primary hepatocytes from calves were treated with various concentrations of NEFA (0, 0.6, 1.2 and 2.4 mM) for the same duration, high concentrations of NEFA significantly elevated the mRNA levels of lipogenesis-related genes, including *DGAT1*, *DGAT2*, *SCD*, and *SREBP1* (Figure 2F).

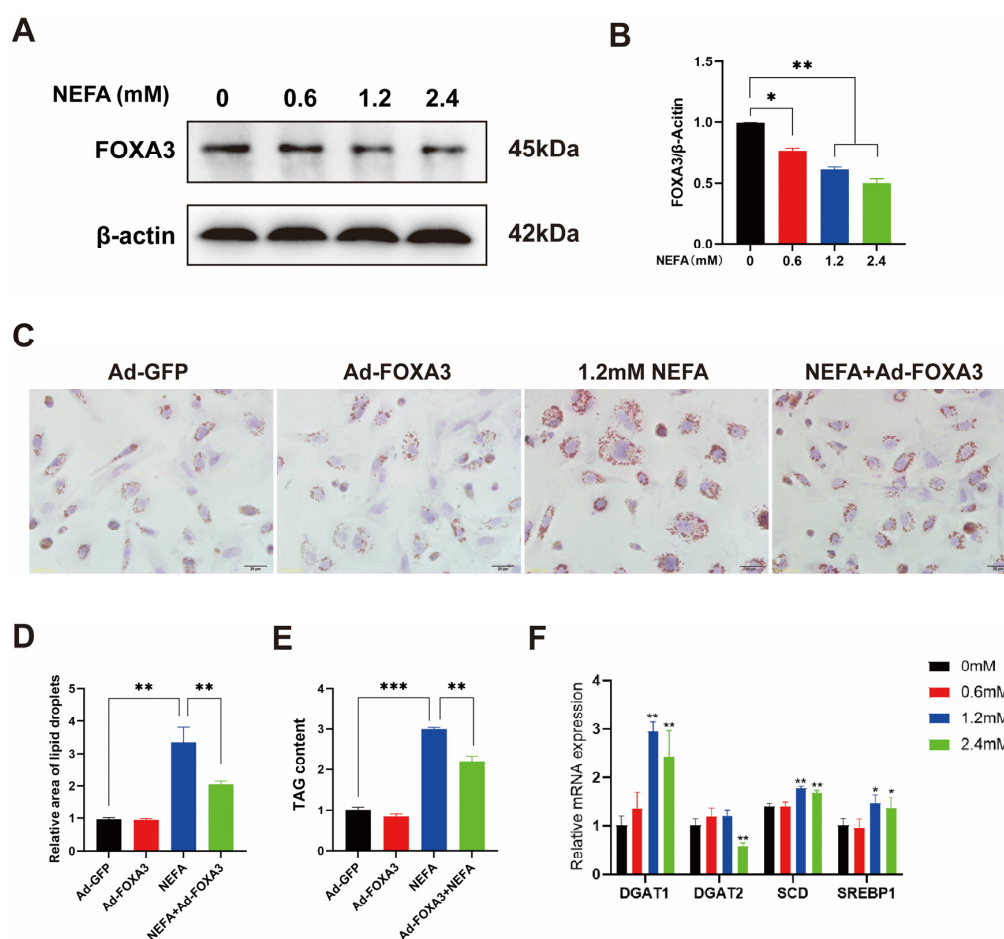


Figure 2. FOXA3 alleviates NEFA-induced lipid accumulation. (A,B) Protein expression and grayscale value quantification of FOXA3 and β-actin in bovine primary hepatocytes treated with different concentrations of NEFA (0.6, 1.2, 2.4 mM). (C,D) Oil Red O staining images and quantitative analysis results of gray values obtained from primary bovine hepatocytes transfected with empty Ad-GFP or Ad-FOXA3 alone for 48 h, or transfected first followed by treatment with 1.2 mM NEFA for 12 h. Images were captured at an original magnification of 40× (scale bar = 20 μm). (E) The triglyceride (TAG) content measured in primary bovine hepatocytes under the following treatments: transfected with Ad-GFP or Ad-FOXA3 alone for 48 h, or transfected first followed by treatment with 1.2 mM NEFA for 12 h. (F) Changes in mRNA abundance of lipogenesis-related genes in bovine primary hepatocytes treated with different concentrations of NEFA. Each experiment was repeated three times. Data were analyzed using one-way ANOVA followed by Bonferroni correction and are presented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. FOXA3 Suppresses the Expression of SREBP1 and Related Lipid Synthesis Genes

Following *FOXA3* overexpression, the protein abundance of SREBP1 and ACC α was significantly downregulated (Figure 3A–D). Concurrently, the mRNA levels of *SREBP1*, fatty acid binding protein 1 (*FABP1*), and ACC α were also significantly reduced (Figure 3E). In contrast, silencing *FOXA3* resulted in elevated mRNA levels of *SREBP1*, *FABP1*, and ACC α compared to the control group (Figure 3F). Additionally, immunofluorescence staining revealed that *FOXA3* overexpression led to an increase in the overall fluorescence intensity of FOXA3 in the cells, indicating elevated expression levels (Figure 3G,H). Conversely, the overall fluorescence intensity of SREBP1 decreased, reflecting reduced expression levels of *SREBP1* (Figure 3I,J).

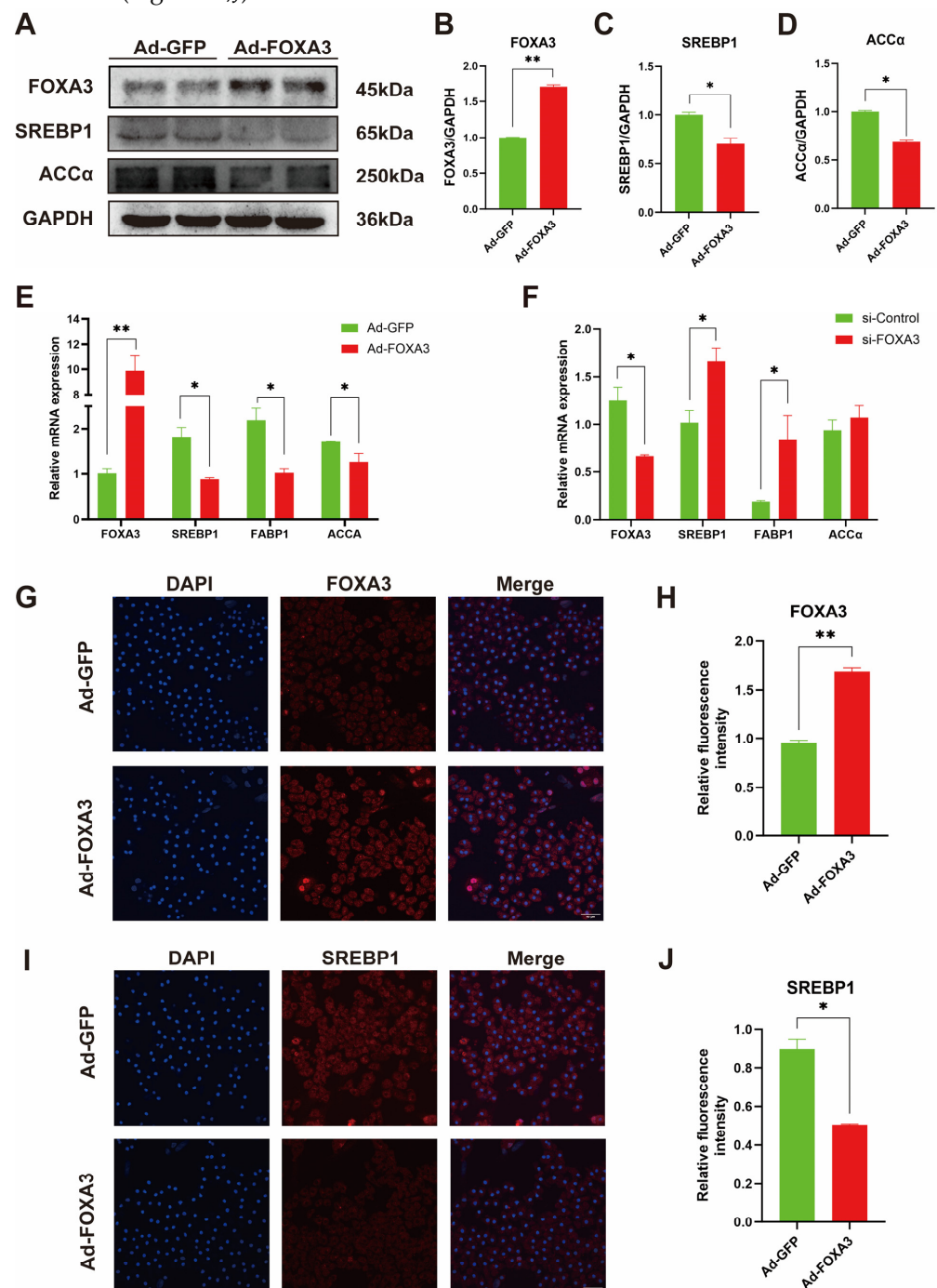


Figure 3. FOXA3 suppresses the expression of SREBP1 and related lipid synthesis genes. (A–D) Protein expression and grayscale value quantification results of FOXA3, SREBP1, and acetyl-CoA carboxylase

alpha (ACC α) in bovine primary hepatocytes 48 h after transfection with Ad-FOXA3 or Ad-GFP. (E) Changes in mRNA abundance of *FOXA3*, *SREBP1*, *ACC α* , and fatty acid binding protein 1 (*FABP1*) in bovine primary hepatocytes after transfection with Ad-GFP or Ad-FOXA3. (F) Changes in mRNA abundance of *FOXA3*, *SREBP1*, *ACC α* , and *FABP1* in bovine primary hepatocytes after transfection with si-Control or si-FOXA3. (G,H) Representative immunofluorescence staining images of FOXA3 and corresponding quantitative analysis of fluorescence intensity obtained from primary bovine hepatocytes transfected with Ad-FOXA3 or Ad-GFP for 48 h, respectively. Original magnification 40 $\times$, scale bar = 40 μ m. (I,J) Representative immunofluorescence staining images of *SREBP1* and corresponding quantitative analysis of fluorescence intensity obtained from primary bovine hepatocytes transfected with Ad-FOXA3 or Ad-GFP for 48 h, respectively. Original magnification 40 $\times$, scale bar = 40 μ m. Blue: nuclei stained with DAPI; Red: FOXA3/*SREBP1*; Merge: co-localization of the indicated proteins. Each experiment was repeated three times. Data were analyzed using independent samples *t*-test followed by Bonferroni correction and are presented as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

3.4. FOXA3 Inhibits Cell Proliferation

Following *FOXA3* overexpression, flow cytometry results indicated a significant reduction in the duration of the S phase and G2/M phase, while the G0/G1 phase was significantly prolonged, leading to a marked decrease in cell proliferation activity (Figure 4B–D). Additionally, qPCR results demonstrated that the mRNA levels of *Cyclin B2* and *Cyclin D2* were significantly reduced after *FOXA3* overexpression.

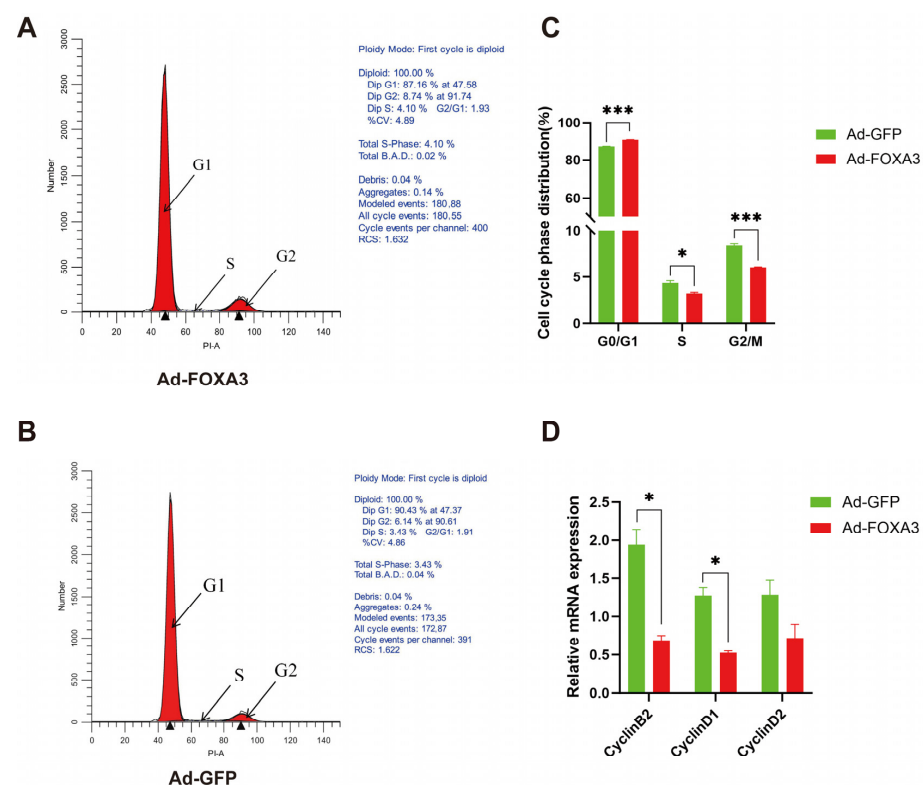


Figure 4. FOXA3 Inhibits Cell Proliferation. (A,B) The percentage of cells in each cell cycle phase was determined by flow cytometry at 48 h after transfection with Ad-FOXA3 or Ad-GFP. (C) Quantitative analysis results of the cell cycle. (D) Changes in mRNA abundance of cell cycle-related genes in bovine primary hepatocytes after Ad-FOXA3 or Ad-GFP transfection. Each experiment was repeated three times. Data were analyzed using independent samples *t*-test followed by Bonferroni correction, and the results were expressed as mean $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$.

3.5. Transcriptome Sequencing of Bovine Primary Hepatocytes with FOXA3 Overexpression

To investigate the potential mechanisms by which *FOXA3* alleviates lipid accumulation, we conducted RNA-seq on primary hepatocytes from the Ad-Control and Ad-FOXA3

groups. Data analysis revealed a total of 378 differentially expressed genes (DEGs), comprising 183 upregulated and 195 downregulated genes (Supplementary Figure S1).

Using the Gene Ontology (GO) database (<http://www.geneontology.org/>), we performed functional annotation of all DEGs, revealing significant enrichment in three major categories (Figure 5). Specifically, the DEGs were significantly enriched in biological processes such as the positive regulation of cell population proliferation and G protein-coupled receptor signaling pathways. They also showed significant enrichment in cellular components, including the nucleus and membrane, as well as in molecular functions such as protein binding and G protein-coupled receptor activity.

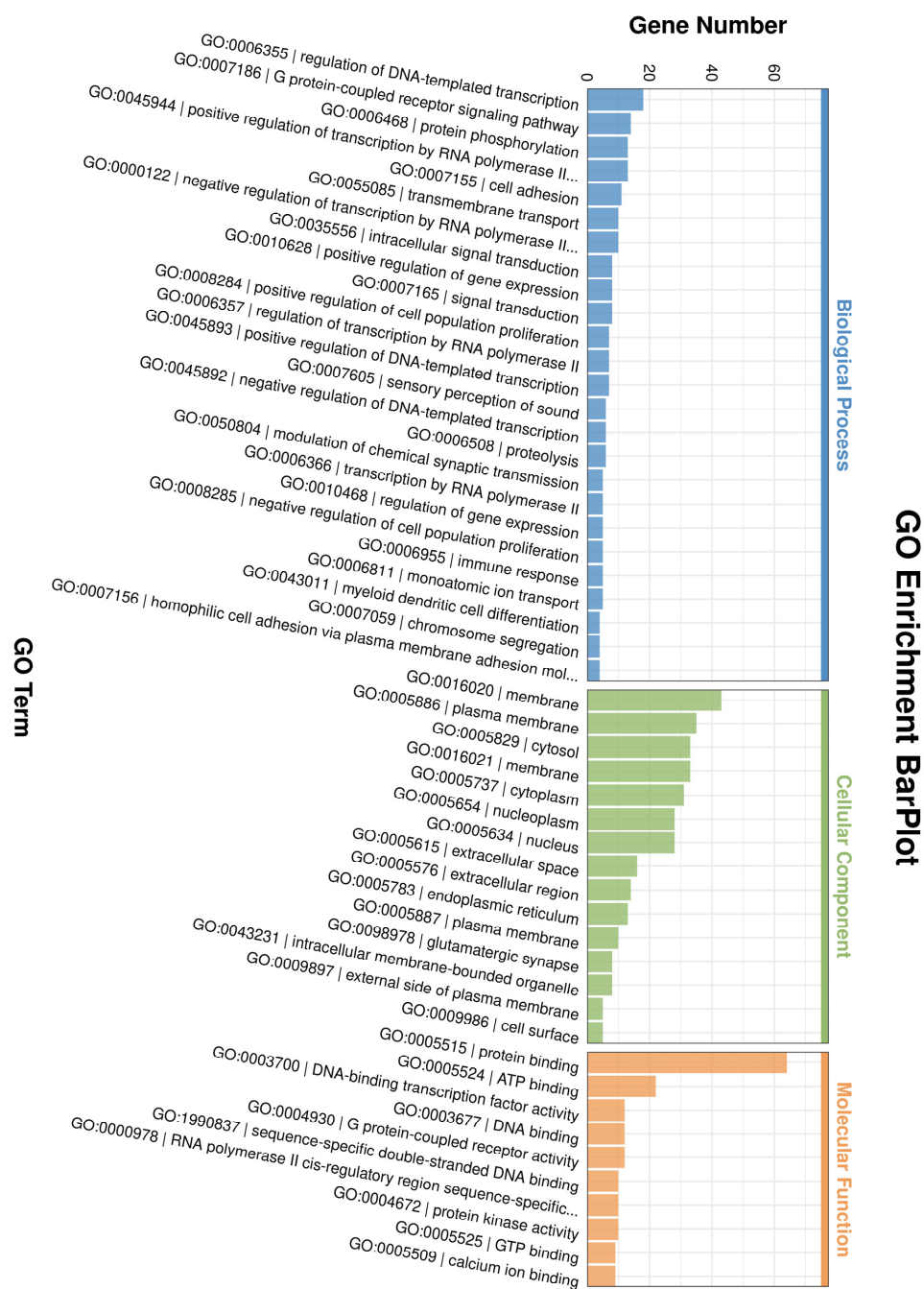


Figure 5. GO enrichment analysis of DEGs. GO:0045944 positive regulation of transcription by RNA polymerase II; GO: 0000122 negative regulation of transcription by RNA polymerase II; GO:0007156 homophilic cell adhesion via plasma membrane adhesion molecules; GO:0000978 RNA polymerase II cis-regulatory region sequence-specific DNA binding.

To further explore the potential functional pathways associated with the DEGs, we conducted KEGG pathway analysis and found significant enrichment in the PI3K-Akt signaling pathway, cell cycle, and arachidonic acid metabolism signaling pathways (Figure 6). Additionally, within the pathways of cholesterol metabolism and cytochrome P450-mediated metabolism of xenobiotics, we identified 3 to 7 downregulated DEGs, with no upregulated DEGs present.

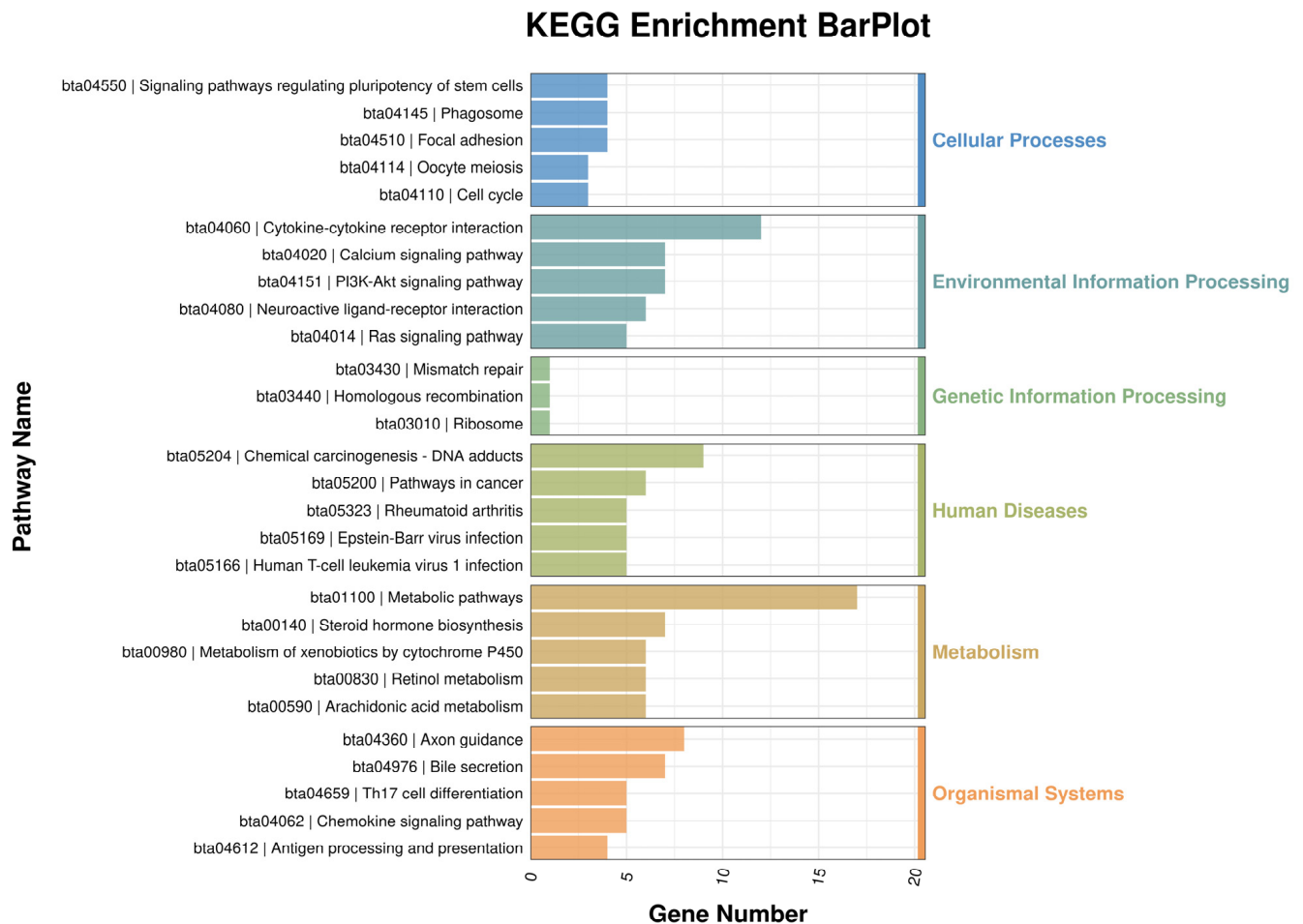


Figure 6. KEGG enrichment analysis of DEGs.

3.6. Validation of Differential Genes

To verify the reliability of the RNA-seq data, we selected the differential genes *NID*, *CACNA2D*, *PTGS2*, *TMEM45B*, *AFP*, *HK2*, and *MYB* for qPCR validation. The results demonstrated that *FOXA3* overexpression resulted in increased expression of the *NID*, *CACNA2D*, *PTGS2*, and *TMEM45B* genes, while the expression of *AFP*, *HK2*, and *MYB* genes decreased. The mRNA expression trends of these genes were consistent with the RNA-seq results (Figure 7A), confirming that the RNA-seq data in this study are accurate and reliable for subsequent analyses.

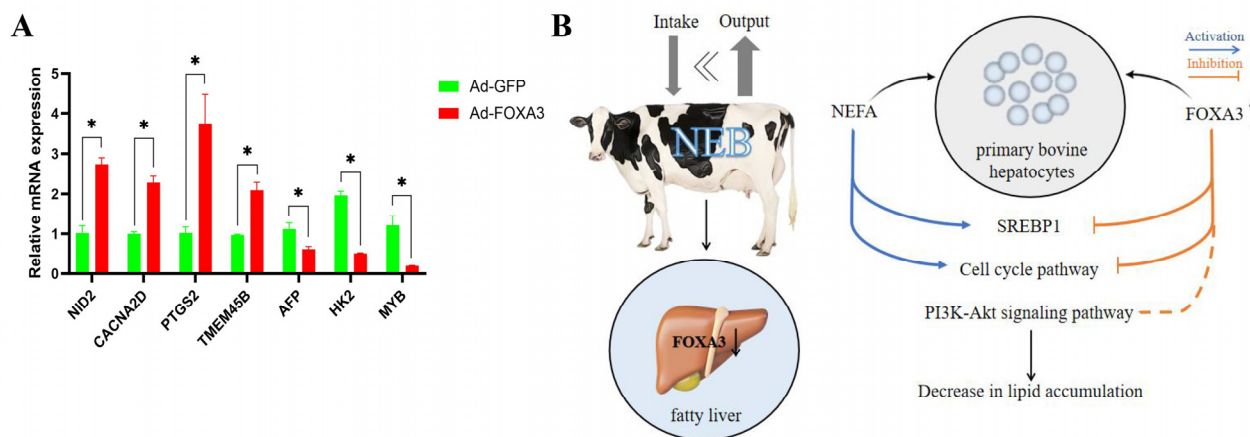


Figure 7. Validation of DEGs and the regulatory mechanism of *FOXA3*. **(A)** qPCR validation results of DEGs. * $p < 0.05$. **(B)** Schematic diagram of the potential regulatory mechanisms of *FOXA3* in lipid deposition of hepatocytes. Blue lines represent promotion, and orange lines represent inhibition; dashed lines indicate pathways that have not yet been clearly defined.

4. Discussion

Numerous studies have demonstrated that *FOXA3* is essential for maintaining lipid and glucose balance in various tissues, including the liver [12,15,27,37]. Previous research indicates that *FOXA3* expression is significantly reduced in the livers of obese or MASH mice [12]. Similarly, in human patients, *FOXA3* expression is downregulated in hepatocellular carcinoma (HCC) cases [17]. These findings suggest a correlation between *FOXA3* expression levels and hepatic metabolic health, with lower *FOXA3* expression observed in livers exhibiting poor metabolic conditions. Our results align with this observation, as *FOXA3* expression is lower in the livers of dairy cows with fatty liver disease compared to healthy cows. Additionally, the FPKM values from RNA-seq indicated that *FOXA3* expression in the livers of cows experiencing severe NEB was lower than in those with mild NEB. Collectively, these experimental results demonstrate that *FOXA3* plays a significant role in the progression of liver injury in both non-ruminant species and dairy cows. While a connection between *FOXA3* and metabolic liver disorders exists, its precise function remains poorly understood.

Excessive exogenous NEFA induce lipid accumulation in bovine hepatocytes, increasing TAG content and elevating the abundance of lipid synthesis genes [38]. This study observed a similar phenomenon, with a marked increase in TAG levels and the number of lipid droplets in primary calf hepatocytes following exposure to 1.2 mM NEFA. Furthermore, the abundance of genes associated with lipid synthesis, including *SREBP1*, was significantly elevated. Notably, our study revealed a decrease in the protein and mRNA abundance of *FOXA3* during lipid accumulation induced by high concentrations of exogenous NEFA, which aligns with findings from in vivo experiments on dairy cows. However, further research is necessary to elucidate the specific role of *FOXA3* in mitigating lipid accumulation in the primary hepatocytes of calves and to clarify the precise regulatory mechanisms involved.

To clarify the influence of *FOXA3* on NEFA-induced lipid accumulation in primary calf hepatocytes, we conducted gain-of-function and loss-of-function experiments. Our results indicate that *FOXA3* overexpression significantly reduced TAG levels caused by NEFA and increased the mRNA abundance of *SREBP1* and *ACCα*. Conversely, silencing *FOXA3* significantly increased the mRNA abundance of *SREBP1* and *FABP1*. Moreover, primary calf hepatocytes with substantial lipid accumulation induced by NEFA exhibited a marked reduction in lipid droplet accumulation following *FOXA3* overexpression, further

supporting our findings. These trends are consistent with observations made in mice models [13]. In summary, these results suggest that *FOXA3* could serve as a significant therapeutic target for reducing lipid accumulation and enhancing liver health in dairy cows.

Experimental data indicate that upregulation of *SREBP1* expression is often accompanied by downregulation of *FOXA3* expression. In ob/ob mice (diabetic and obese models) and db/db mice (type 2 diabetic models), *SREBP1* expression is upregulated by more than two-fold, whereas hepatic *FOXA3* protein and transcript abundance are reduced by over 50% [12]. Our experimental results support this observation; following *FOXA3* overexpression, both the mRNA and protein expression levels of *SREBP1* were significantly reduced. Immunofluorescence staining further revealed that after overexpression, the fluorescence intensity of *FOXA3* increased, indicating elevated *FOXA3* expression, while the fluorescence intensity of *SREBP1* decreased, demonstrating reduced *SREBP1* expression. These results suggest that *FOXA3* may inhibit *SREBP1* activation or promote its degradation. Additionally, *FOXA3* may influence the subcellular localization of *SREBP1* and indirectly affect its activity through specific pathways, thereby impacting lipid synthesis. A previous study has established a potential link between *SREBP1* and *FOXA3*, indicating that *FOXA3* can influence hepatic lipid synthesis and steatosis in mice via the XBP1s-*FOXA3*-PER1/*SREBP1c* axis [27]. However, there is currently no direct literature confirming that *FOXA3* can regulate *SREBP1* localization.

One study demonstrated that the expression levels of Cyclin B and Cyclin D are significantly associated with postpartum ketosis in dairy cows [39]. Additionally, research has shown that elevated *FOXA3* expression enhances hepatocyte function while inhibiting cell cycle progression by downregulating Cyclin-dependent kinase 4 (*CDK4*), *Cyclin D1*, and *Cyclin E1*, thereby suppressing cell proliferation. Flow cytometry results from this study demonstrated an increased proportion of cells in the G1 phase, confirming the blockage of the cell cycle [40]. Therefore, *FOXA3* may play a dual role in promoting liver function while inhibiting proliferation by regulating cell cycle-related factors. This conclusion is supported by immunohistochemical and transplantation experiment results [40]. Our flow cytometry results also indicated that following *FOXA3* overexpression, the G1 phase was prolonged, while the S and G2 phases were shortened. qPCR results showed that the mRNA expression levels of *Cyclin D1* and *Cyclin B1* were significantly downregulated, suggesting a reduction in cell proliferation activity. Additionally, our other experimental results demonstrated that *FOXA3* could reduce lipid accumulation in the liver and improve liver metabolism. We speculate that the decline in cell proliferation may reduce the cellular demand for lipids while enhancing lipid metabolism, thereby alleviating the phenotype of excessive lipid accumulation. Although we hypothesize that *FOXA3* is actively involved in hepatic lipid synthesis and metabolism by influencing the localization and expression of *SREBP1*, as well as cell cycle progression, further studies are needed to clarify the precise mechanisms through which *FOXA3* acts during liver development. To gain deeper insights into the potential mechanisms by which *FOXA3* regulates lipid synthesis and metabolism, we performed RNA-seq on cells from both the control group and the *FOXA3*-overexpressing group. The sequencing results indicated that *FOXA3* may exert its effects by influencing pathways such as the arachidonic acid metabolism pathway and the PI3K-Akt signaling pathway. The PI3K-Akt pathway is essential for regulating lipid and protein metabolism and is also associated with cell proliferation [41]. A high-fat diet has been shown to inhibit the PI3K-Akt pathway [42], while activation of this signaling pathway in the liver can alleviate systemic insulin resistance and obesity [43]. The PI3K-Akt signaling cascade directly affects glucose metabolism by phosphorylating key metabolic enzymes, and the biological effects of insulin are dependent on this pathway [44]. Furthermore, lipid metabolism is closely linked to atherosclerosis and insulin resistance. Abnormal lipid metabolism can

trigger lipotoxicity, leading to inflammatory responses in tissues and organs [45]. Conversely, inflammation inhibits insulin signal transduction, ultimately inducing insulin resistance [46]. Given these associations, it can be concluded that the PI3K-Akt signaling pathway plays a crucial role in lipid metabolism. In our RNA-seq results, we found that after *FOXA3* overexpression, the expression of *MYB* differentially expressed gene enriched in the PI3K-Akt pathway was downregulated, while the expressions of *ITGA11* and *LAMA3* were upregulated. Arachidonic acid metabolism regulates hepatic glucose and lipid homeostasis through three major pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450). Dysregulation of these pathways is closely associated with various metabolic diseases. Our data reveal upregulation of prostaglandin-endoperoxide synthase 2 (*PTGS2*, also known as *COX-2*), an enzyme involved in lipid peroxidation and the generation of bioactive lipids [47]. Therefore, *FOXA3* may influence hepatic lipid synthesis via the PI3K-Akt and arachidonic acid metabolism pathways; however, this hypothesis requires further experimental validation.

The KEGG enrichment results indicate that pathways related to the cell cycle and cell proliferation are significantly enriched. Disruption of the balance among CDKs, cyclins, and CDK inhibitors can adversely affect cell proliferation and division. Additionally, differential gene expression analysis reveals that under conditions of *FOXA3* overexpression, the expression of key cell cycle proteins, such as CDK1 and CCNE1, which promote the transition from the G1 to S phase and from the G2 to M phase, is downregulated. These findings provide a robust molecular explanation at the transcriptomic level for the G0/G1 phase arrest induced by *FOXA3*, corroborated by flow cytometry analysis.

RNA-seq data indicated that the expression of *TMEM45B*, a gene associated with pathways related to integral membrane components, was upregulated. Studies have demonstrated that in pigs with non-alcoholic steatohepatitis, squalene intake reduces lipid droplet synthesis and alleviates steatosis. The RNA-seq results showing increased expression of *TMEM45B* align with our findings [48]. Additionally, the downregulation of the key glycolytic gene *HK2* indicates that *FOXA3* may accelerate lipid oxidation by inhibiting glycolysis. Moreover, the expression of the *PLIN4* gene in the PPAR signaling pathway was found to be downregulated. *PLIN4* is not only a downstream target of *PPAR* γ [49–51] but also a specific protein induced during adipocyte differentiation [52]. Previous studies have shown that *FOXA3* is located upstream of *PPAR* γ , suggesting that *FOXA3* may downregulate the expression of *PLIN4* through *PPAR* γ [31], thereby affecting its efficiency in lipid droplet biogenesis. These findings provide a novel mechanistic perspective on how *FOXA3* alleviates lipid accumulation. While the RNA-seq analysis in this study offers a wealth of candidate targets, further experimental validation is necessary to determine whether *FOXA3* directly or indirectly regulates these DEGs.

5. Conclusions

This study demonstrates that cows with clinical ketosis exhibit severe hepatic lipid accumulation and reduced *FOXA3* abundance; *FOXA3* is downregulated by high concentrations of exogenous NEFA in bovine primary hepatocytes. Notably, overexpression of *FOXA3* alleviates NEFA-induced lipid accumulation, whereas silencing *FOXA3* exacerbates lipid deposition. Furthermore, *FOXA3* influences lipid synthesis by affecting the expression levels and cellular localization of *SREBP1*, or through potential signaling pathways (Figure 7B). These findings suggest that *FOXA3* overexpression could serve as a potential therapeutic strategy to mitigate hepatic lipid accumulation in cows with ketosis or fatty liver.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/vetsci13020157/s1>, Figure S1: Differentially expressed genes in different groups; Table S1: The siRNA sequence used during hepatocytes transfection; Table S2: Primer sequences used for quantitative real-time PCR.

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Abbreviations

The following abbreviations are used in this manuscript:

NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
TAG	Triacylglycerol
FOXA3	Forkhead Box A3
HCC	Hepatocellular Carcinoma
SREBP1	Sterol Regulatory Element-Binding Protein 1
BSA	Bovine Serum Albumin
Ad-GFP	Adenovirus-Green Fluorescent Protein
Ad-FOXA3	Adenovirus-Forkhead Box A3
siRNA	small interfering RNA
si-Control	small interfering RNA-Control
si-FOXA3	small interfering RNA-FOXA3
DEGs	Differentially Expressed Genes
CDK	Cyclin-Dependent Kinase
COX	Cyclooxygenase
LOX	Lipoxygenase
CYP450	Cytochrome P450
PTGS2	Prostaglandin-Endoperoxide Synthase 2

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