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Research Article

The effects of hepatocyte-specific MafF overexpression on FFA or ETOH induced hepatocyte steatosis and its underlying mechanism

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Running head: Effects of MafF on hepatocyte steatosis

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Abstract

Background and Aim: The transcription factor MafF is a novel regulator of adipogenesis, but its role in hepatic steatosis remains unclear. This study aimed to explore the impact of MafF on hepatocyte steatosis and its underlying mechanisms.

Materials and Methods: A stable MafF-overexpressing cell line was established using lentiviral infection. RT-qPCR and Western blot analysis confirmed MafF expression. Free fatty acids (FFA) or ethanol (ETOH) induction was used to simulate hepatocyte steatosis in non-alcoholic or alcoholic fatty liver disease (NAFLD or AFLD). Cell activity and lipid accumulation were assessed through CCK-8 assay, Calcein-AM/PI staining, and oil red O staining. The changes in lipid metabolism-related gene expression before and after FFA or ETOH treatment were detected using RT-qPCR.

Results: FFA or ETOH-induced lipid accumulation in hepatocytes, and overexpression of MafF significantly ameliorated ETOH-induced hepatocyte steatosis, but had little effect on FFA-induced hepatocyte steatosis. MafF overexpression could significantly reduce the expression of peroxisome proliferator-activated receptor Gamma (PPARG), acetyl-CoA carboxylase (ACC), and lipoprotein lipase (LPL) in hepatocytes. Upon FFA induction, control (NC) cells exhibited down-regulation of these genes, whereas MafF-overexpressing cells up-regulated LPL expression. In contrast, under ETOH treatment, NC cells up-regulated these genes, while MafF-overexpressing cells showed a down-regulation.

Conclusion: This study highlighted the regulation of lipid-related genes by MafF, including PPARG, ACC, and LPL, and affected FFA and ETOH-induced hepatocellular lipid accumulation in distinct ways, and MafF showed a more pronounced improvement in ETOH-induced hepatocyte steatosis, providing crucial insights into MafF's role in hepatic lipid metabolism and potential therapeutic strategies for NAFLD and AFLD.

Keywords: ACC; Hepatocyte steatosis; LPL; MafF; PPARG.

Introduction

Excessive fat storage and steatosis in hepatocytes, driven by disorders of fat metabolism, are central to the development of liver diseases, such as non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD)^[1]. Both NAFLD and AFLD share similar pathological features, including disrupted lipid metabolism and intracellular lipid accumulation in lipid droplets, ultimately leading to fatty liver^[2,3]. However, their etiologies differ. NAFLD is primarily caused by excessive dietary fat intake, which leads to increased de novo lipid synthesis and reduced fatty acid β -oxidation in the liver^[4]. In contrast, AFLD results from chronic heavy alcohol consumption, which impairs lipid oxidation and degradation^[5]. While the progression from simple fatty liver to steatohepatitis can be reversed, further advancement can lead to irreversible cirrhosis and liver cancer, highlighting the critical importance of prevention and treatment of fatty liver.

MafF (v-maf musculoaponeurotic fibrosarcoma oncogene homolog F) is a small Maf

protein belonging to the bZIP transcription factor family. It forms homo- or heterodimers with other sMaf proteins or bZIP transcription factors such as Nrf2 (NF-E2-related factor 2), CNC (Cap'n'collar), and Bach^[6]. MafF is implicated in various biological processes, including cell cycle regulation^[7], proliferation^[8], apoptosis^[9], oxidative stress^[10], inflammation^[11], autophagy^[12], drug resistance^[13], and carcinogenesis^[14].

Recently, MafF has been identified as a novel regulator of adipogenesis and plays a crucial role in atherosclerosis and cholesterol metabolism by influencing antioxidation and regulating LDLR gene expression^[9,15,16]. LDLR is a key gene in regulating LDL and blood cholesterol balance. MafF can induce LDLR expression under non-inflammatory conditions but decrease it under inflammation or a high-fat diet, thereby increasing cardiovascular risk^[15]. Additionally, MafF has been shown to protect arterial endothelial cells from damage and apoptosis caused by palmitic acid^[9]. The Activating Transcription Factor 3 (ATF3) can indirectly regulate LDLR expression through MafF and is involved in lipid metabolism, inflammation, and atherosclerosis^[16]. These studies suggest that MafF is closely related to lipid metabolism. However, the specific mechanisms and the relationship between MafF and lipid metabolism-related diseases such as NAFLD and AFLD require further investigation. This study aims to explore the effect of MafF on hepatocyte steatosis and its relationship with lipid metabolism-related genes, such as Peroxisome Proliferator Activated Receptor Gamma (PPARG)^[17,18], Acetyl CoA Carboxylase (ACC or ACACA)^[19,20], Lipoprotein Lipase

(LPL)^[21,22], to provide new clues for further mechanism research, prevention, and treatment of NAFLD and AFLD.

Materials and Methods

Cells and Reagents

SMMC7721 cells were obtained from iCell Bioscience Inc. DMEM/F12 medium, fetal bovine serum, and penicillin/streptomycin mixture were purchased from Pricella. BCA protein assay kit, Oil Red O staining kit, Calcein/PI cell viability/cytotoxicity assay kit, and CCK-8 kit were purchased from Beyotime. Oleic acid and palmitic acid were acquired from Sigma-Aldrich. MafF antibody was purchased from Proteintech. β -actin antibody, Goat Anti-Rabbit IgG (H+L) HRP were purchased from Affinity. RNAiso Plus (Trizol), PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), and TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara.

Since no human subjects were involved in this study, the Ethics Committee Approval and the Declaration of Helsinki are not required.

Stable Overexpression Cell Line Construction

The sequence of the human MafF gene (Gene ID 23764) transcript (NM_012323.4) was obtained from the NCBI website (www.ncbi.nlm.nih.gov), and the full-length protein coding sequence (495 bp) was synthesized by Sangon Biotech, Shanghai, China. This MafF coding sequence was cloned into a lentiviral expression vector, and the

plasmid pLVX-mCherry-MafF was confirmed by DNA sequencing. After lentiviral packaging and infection of SMMC7721 cells, a stable overexpression cell line, SMMC7721-MafF, was generated through selection with 10 μ g/ml puromycin for one week. A vector control cell line, SMMC7721-NC, was also established. Both stable cell lines were maintained in culture with 2 μ g/ml puromycin.

Protein Extraction and Western Blot

Cells were lysed with RIPA lysis buffer to extract total protein, followed by quantification using a BCA protein assay kit. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking with 5 % skimmed milk, the membranes were incubated with the MafF primary antibody, followed by incubation with the secondary antibodies. After washing the membranes 3 times with TBST, ECL luminescent solution was added for chemiluminescent detection. β -actin served as the loading control.

Cell Culture and Induction Treatment

Cells were cultured in DMEM/F12 complete medium (DMEM/F12 medium supplemented with 1 % penicillin/streptomycin, 10 % fetal bovine serum, and 2 μ g/ml puromycin) at 37 °C in a 5 % CO₂ atmosphere. A 7.5 mM stock solution of free fatty acid (FFA, oleic acid: palmitic acid = 2:1) was prepared. After normal overnight culture, the DMEM/F12 complete medium was replaced with FFA-containing or ETOH-containing medium, and then cells were cultured for 24 hours for induction treatment.

Cell Viability Assay

Cells were seeded at a density of 5,000 cells per well in 96-well plates. Cell viability was assessed using CCK-8 kit or Calcein-AM/PI cell viability/cytotoxicity assay kit. For CCK-8 assay, cells were incubated with reaction solution for 4 h, and the absorbance was measured at 450 nm. For Calcein-AM/PI assay, cells were incubated with the reaction buffer for 30 min, and live/dead cells were visualized under a fluorescence microscope.

Oil Red O Staining

Cells were fixed with 1 ml fixative solution per well for 10 min, then stained with oil red O working solution at room temperature for 20 min. After washing twice with the washing solution, lipid droplets in cells were observed under a microscope.

RNA Extraction and RT-qPCR

Total RNA was extracted from cells using Trizol reagent, and RNA purity and concentration were measured using a micro nucleic acid detector. Reverse transcription and quantitative amplification reactions were performed according to the manufacturer's instructions (TaKaRa). The $2^{-\Delta\Delta CT}$ method was used to evaluate the relative mRNA expression level of target genes. The primer sequences, synthesized by Sangon Biotech (Shanghai) Co., Ltd., are listed in Table 1. β -actin was used as the internal reference.

Statistical Analysis

The GraphPad Prism 10.0 software was used for statistical analysis. Results were presented as mean \pm SEM. T-tests were used to compare differences between two independent groups, and one-way ANOVA was applied for comparisons among multiple groups. A p-value of <0.05 was considered statistically significant. Representative images were quantified using Image J software.

Results

Successful Construction of MafF Stable Overexpression Cell Line

After plasmid construction, lentiviral packaging, cell infection, and puromycin selection, stable cell lines SMMC7721-MafF and SMMC7721-NC (vector control) were established. RT-qPCR and Western blot results showed that the mRNA and protein expression levels of MafF were significantly increased in SMMC7721-MafF cells compared to NC control cells (Figure 1). These results confirmed the successful construction of the MafF stable overexpression cell line.

MafF Overexpression Reduced the Expression of PPARG, ACC, and LPL

Genes

RT-qPCR was performed to measure the expression of three lipid metabolism-related genes, as shown in Figure 2. In SMMC7721-MafF cells, the mRNA levels of PPARG, ACC, and LPL were significantly reduced compared to NC cells, indicating that MafF

overexpression can dramatically down-regulate these lipid metabolism-related genes.

Effect of MafF on FFA-induced Hepatocyte Steatosis

When treated with increasing concentrations of FFA, both SMMC7721-MafF and SMMC7721-NC cells exhibited decreased viability, as demonstrated by the CCK-8 assay results, which indicated that FFA had a certain level toxicity to cells. After 24 hours of treatment, the IC_{50} of FFA was 2.399 mM for SMMC7721-NC cells and 3.479 mM for SMMC7721-MafF cells, and the higher IC_{50} indicated that SMMC7721-MafF cells have increased resistance to FFA (Figure 3A). Additionally, the Calcein-AM/PI staining demonstrated increased cell death following treatment with 2 mM FFA (Figure 3B), showing a certain cellular toxicity of FFA. Quantitative analysis revealed that the percentage of dead cells increased from 6% to 17% in SMMC-NC cells, and from 5% to 12% in SMMC7721-MafF cells, respectively. The lower cell death rates suggested that MafF overexpression slightly increased cellular resistance to FFA.

Oil Red O staining showed an increase in FFA-induced lipid accumulation in both cells, with a concentration-dependent effect (Figure 3C). However, there was no significant difference in lipid droplet accumulation changes between SMMC7721-MafF and NC cells (Figure 3D). These findings indicate that FFA can induce lipid accumulation in hepatocytes, and MafF overexpression has little effect on FFA-induced hepatocyte steatosis.

Effect of MafF on ETOH-induced Hepatocyte Steatosis

SMMC7721-MafF and SMMC7721-NC cells were exposed to 0%~5% ETOH, and cell viability was assessed using CCK-8 assay after 24 hours. Both cells showed a dose-dependent decrease in viability, with IC₅₀ values of 2.374% for NC cells and 2.271% for MafF cells, indicating a minimal impact of MafF overexpression on ETOH sensitivity (Figure 4A). Calcein-AM/PI staining revealed a slight increase in cell death at 2% ETOH for both cells, from 8% to 13% for NC cells and from 9% to 15% for MafF cells, suggesting that MafF overexpression had little effect on cellular sensitivity to ETOH (Figure 4B).

Oil Red O staining demonstrated that ETOH induced dose-dependent lipid accumulation in both cells (Figure 4C). Notably, MafF overexpression significantly reduced lipid droplet formation compared to NC cells, with a reduction from 20.13% to 12.21% at 2% ETOH, and from 34.95% to 21.52% at 5% ETOH (Figure 4D). These findings suggest that MafF overexpression mitigates ethanol-induced steatosis in hepatocytes while having minimal impact on ethanol sensitivity.

Effect of MafF on the Regulation of PPARG, ACC, and LPL genes During Hepatocyte Steatosis

Based on the CCK-8, Calcein-AM/PI staining, and Oil Red O staining results, 2 mM FFA and 2% ETOH were used to induce hepatocyte steatosis in subsequent research. RT-qPCR analysis showed a significant decrease in the expression of PPARG, ACC, and LPL genes in SMMC7721-NC cells after FFA induction (Figure 5A-C). In

SMMC7721-MafF cells, PPARG expression decreased further (*P < 0.05), ACC remained unchanged, and LPL switched from downregulation to significant upregulation (****P < 0.0001), indicating that MafF overexpression substantially affects LPL gene expression during FFA induction. MafF overexpression decreases PPARG expression but increases LPL expression after FFA induction, potentially counteracting their effects in hepatocytes. However, the situation was significantly different after ETOH induction. RT-qPCR results demonstrated a conversed upregulated expression of PPARG and LPL genes in NC cells (**P < 0.01), while a significantly reduced expression of all three genes in SMMC7721-MafF cells (**P < 0.001, ****P < 0.0001), which may result in a significant improvement in lipid accumulation in hepatocytes (Figure 5D-F).

Discussion

The liver is the primary site for lipid metabolism and plays a crucial role in lipid synthesis, breakdown, and transport. NAFLD and AFLD are two major chronic liver diseases closely related to lipid metabolism disorders, but many aspects of their pathogenesis remain controversial^[23]. The transcription factor MafF is a newly identified lipogenesis regulator, but its relationship to lipid metabolism and associated diseases such as NAFLD and AFLD is not fully understood. In this study, we induced hepatocyte steatosis with FFA or ETOH to mimic NAFLD and AFLD, respectively. We explored the effects of MafF on hepatocyte steatosis and possible regulatory

mechanisms. We found that MafF overexpression reduced the expression of lipid metabolism-related genes PPARG, ACC, and LPL. Interestingly, the expression levels of these genes varied between FFA-induced and ETOH-induced conditions. Moreover, MafF exhibited distinct roles in each process, exerting a more pronounced effect on ETOH-induced hepatocyte steatosis.

Lipid metabolism is a complex process regulated by various mechanisms. PPARG, a key regulator of lipogenesis and fat tissue development, promotes adipocyte differentiation and lipid storage, which are crucial for glycolipid processes and inflammation^[17]. ACC, an essential enzyme in fatty acid synthesis, converts Acetyl CoA to Malonyl CoA, the initial and rate-limiting step in fatty acid production^[19,20]. LPL, another vital metabolic enzyme, breaks down triglycerides and very low-density lipoproteins, releasing fatty acids and monoacylglycerols for energy or storage, thereby managing blood lipid levels and energy balance^[21,22]. Our research showed that MafF overexpression downregulates these genes, suggesting that MafF may be involved in lipogenesis and storage processes, and implicate hepatocellular steatosis by transcriptionally regulating the expression of these genes.

Importantly, we found that MafF had different impacts on FFA- and ETOH-induced hepatocyte steatosis, possibly due to varying mechanisms underlying NAFLD and AFLD. In NAFLD, excess FFA intake leads to liver fat accumulation. After FFA induction, the expressions of PPARG, ACC, and LPL were significantly downregulated in NC cells; PPARG expression was further decreased in MafF-overexpressing cells,

suggesting that MafF may alleviate lipid accumulation by downregulating the adipogenesis. However, the expression of the LPL gene in MafF-overexpressing cells changed dramatically from downregulation to significant upregulation, indicating that MafF plays a crucial role in regulating LPL during FFA-induced hepatocyte steatosis. We speculated that MafF could increase lipid accumulation in hepatocytes by upregulating LPL, which hydrolyzes TAG into FFA and glycerol, thus enhancing catabolism and utilization of TAG and leading to subsequent fat accumulation^[24]. Overall, MafF decreases PPARG expression and boosts LPL expression after FFA induction, affecting fatty acid metabolism and lipid deposition without significant difference in lipid accumulation between SMMC7721-MafF and NC cells following FFA induction.

However, after ETOH induction, the expression levels of PPARG and LPL both increased, with a slight elevation in ACC levels. This response differs from FFA induction, indicating distinct regulation mechanisms between NAFLD and AFLD, and maybe different roles of MafF in them. The upregulation of PPARG enhances adipocyte differentiation and lipid storage, while also activating ACC to promote fatty acid synthesis^[18]. Moreover, the elevated expression of LPL further contributes to fat accumulation^[19,20,25]. Consequently, ETOH treatment can significantly increase cellular fat content by upregulating these three genes. This study confirmed the mRNA levels of PPARG, ACC, and LPL, particularly LPL gene, were reduced significantly in MafF-overexpressing cells. We speculate that MafF can downregulate the expression of these

genes after ETOH treatment, thereby reducing fatty acid synthesis and storage, ultimately mitigating ETOH-induced fat accumulation. In addition, MafF overexpression may alter alcohol metabolism or related injury, which could be another regulatory mechanism. Further investigation is needed to explore that whether MafF regulates the expression or activity of key enzymes in alcohol metabolism, such as alcohol dehydrogenase (ADH), aldehyde dehydrogen (ALDH), and cytochrome P450 2E1 (CYP2E1), thereby affecting alcohol metabolism and ultimately leading to hepatocellular damage and fat accumulation. In summary, the regulatory mechanism of MafF improving alcoholic hepatocellular steatosis may be complex, and it is a very worthy direction for in-depth research.

This primary study has for the first time indicated the effects of MafF as an emerging novel lipogenesis regulator on lipid metabolism-related genes and its relationship with hepatocyte steatosis. Further studies are necessary to assess the effects and it would be beneficial to include the evaluation of MafF and related genes in future studies of related diseases, considering their impact on lipid synthesis and metabolism.

Conclusion

This study demonstrates transcription factor MafF is a lipogenesis regulator and it can transcriptionally regulate PPARG, ACC, and LPL genes to reduce lipid synthesis, prevent lipid accumulation in hepatocytes, and ameliorate hepatocyte steatosis. These results lay a robust foundation for further investigation into the role of MafF in hepatic lipid metabolism and related disorders, providing new insights into the prevention and

treatment of NAFLD and AFLD.

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Tables

Table 1. Primers used in this study.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
MafF	GATCCCCTATCCAGCAAAGC	CTTCTGCAGCTCCTCCTTCT
PPARG	AGCCCTTCACTACTGTTGACT	CTTTGATTGCACTTTGGTACT
ACC	GTCAAGAAGAAAATCCACAA	TTGTCCCAAACATAAGCCTT
LPL	GTCCGTGGCTACCTGTCATTT	GGCACCCAACTCTCATACAT
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG

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Figure Legends

Figure 1. Differential expression of MafF in stable cell lines SMMC7721-MafF and SMMC7721-NC. (A) RT-qPCR detected the mRNA expression level of MafF gene; (B) The protein expression level of MafF was detected by Western Blot, with β -actin used as the loading reference. ** $p < 0.01$.

Figure 2. MafF overexpression reduced the expression of the genes related to lipid synthesis and metabolism. RT-qPCR results of (A) PPARG gene, (B) ACC gene, and (C) LPL gene in SMMC7721-MafF and NC cells, with β -actin used as the internal reference. *** $p < 0.001$, **** $p < 0.0001$.

Figure 3. Effect of MafF on FFA-induced hepatocyte steatosis. (A) Cell viability of SMMC7721-MafF and NC cells treated with different concentrations of FFA were detected by CCK-8 assay; (B) Cell deaths were evaluated using Calcein-AM/PI staining after 0 and 2 mM FFA treatment; (C) The intracellular accumulation of lipid droplets after treatment with different concentrations of FFA were observed using Oil red O staining (x200); (D) The positive area ratio of oil red O staining were quantitatively analyzed using Image J software; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Effect of MafF on ETOH-induced hepatocyte steatosis. (A) Cell viability of SMMC7721-MafF and NC cells after treatment with different concentrations of ETOH were detected by CCK-8 assay; (B) Cell death of SMMC7721-MafF and NC cells after 0 and 2 % ETOH treatment were evaluated using Calcein-AM/PI staining; (C) The intracellular accumulation of lipid droplets after treatment with different concentrations of ETOH were observed using Oil Red O staining (x200); (D) The positive area ratio of oil red O staining were quantitatively analyzed using Image J software; *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

Figure 5. Effect of MafF on the expression of PPARG, ACC, and LPL genes during FFA-induced (A-C) and ETOH-induced (D-F) hepatocyte steatosis.

Figures

Figure 1

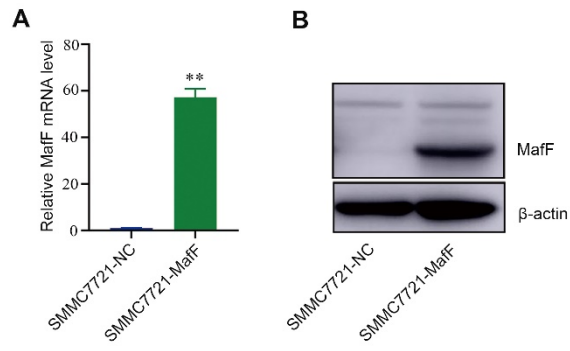


Figure 2

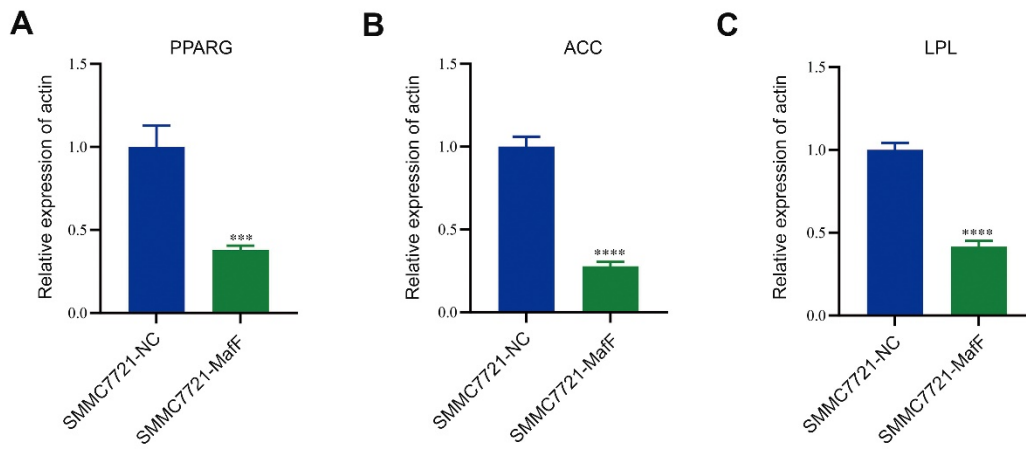


Figure 3

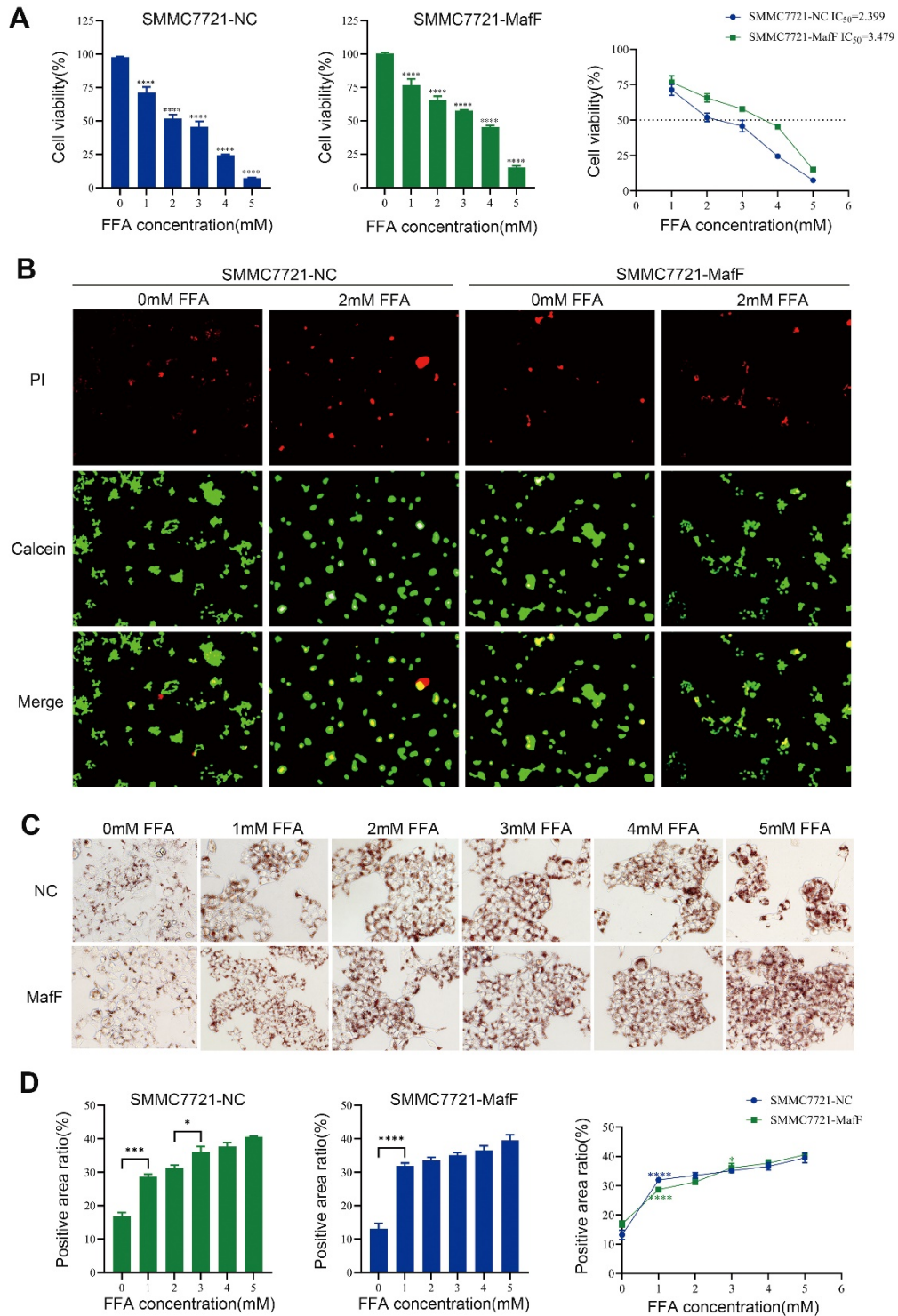


Figure 4

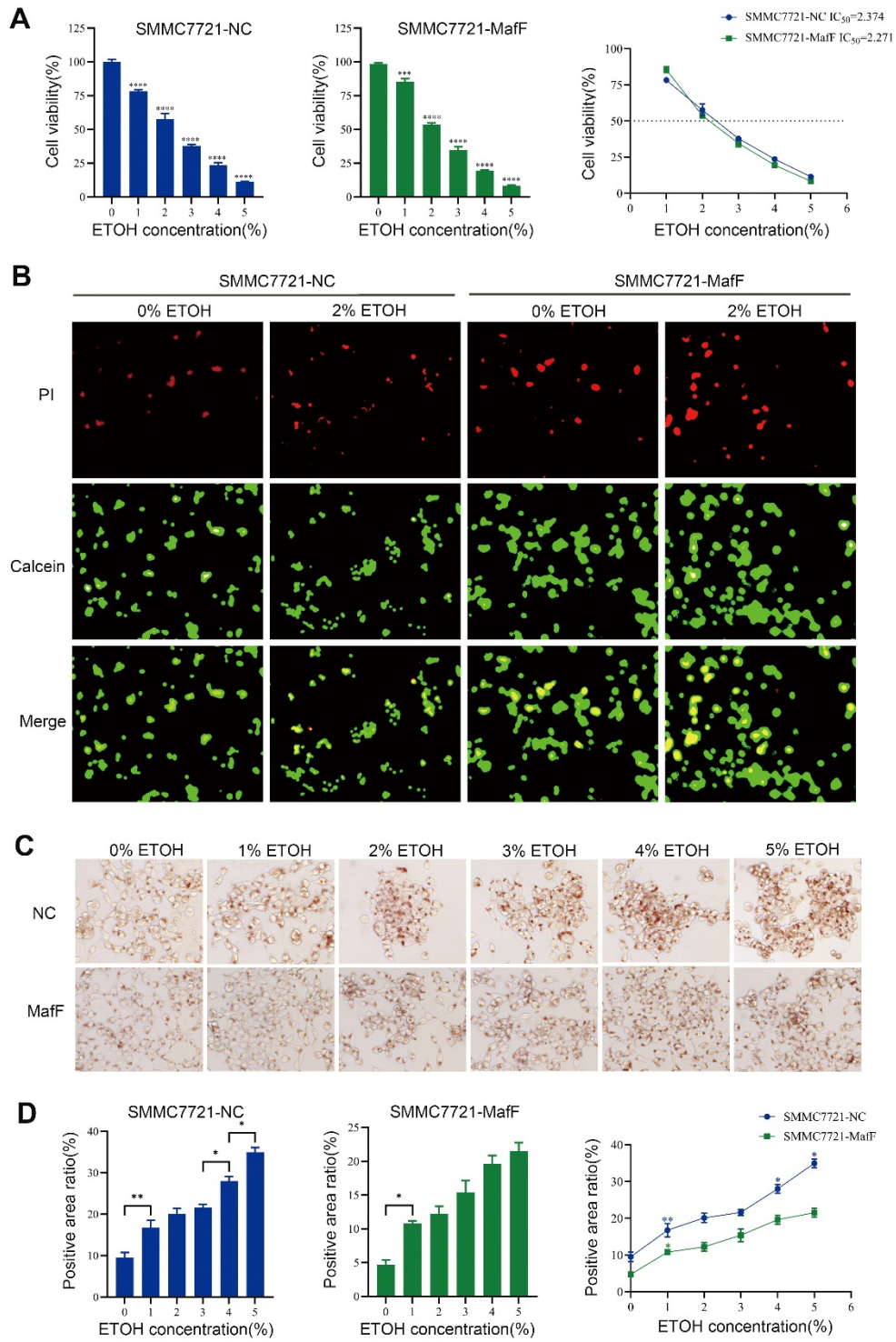
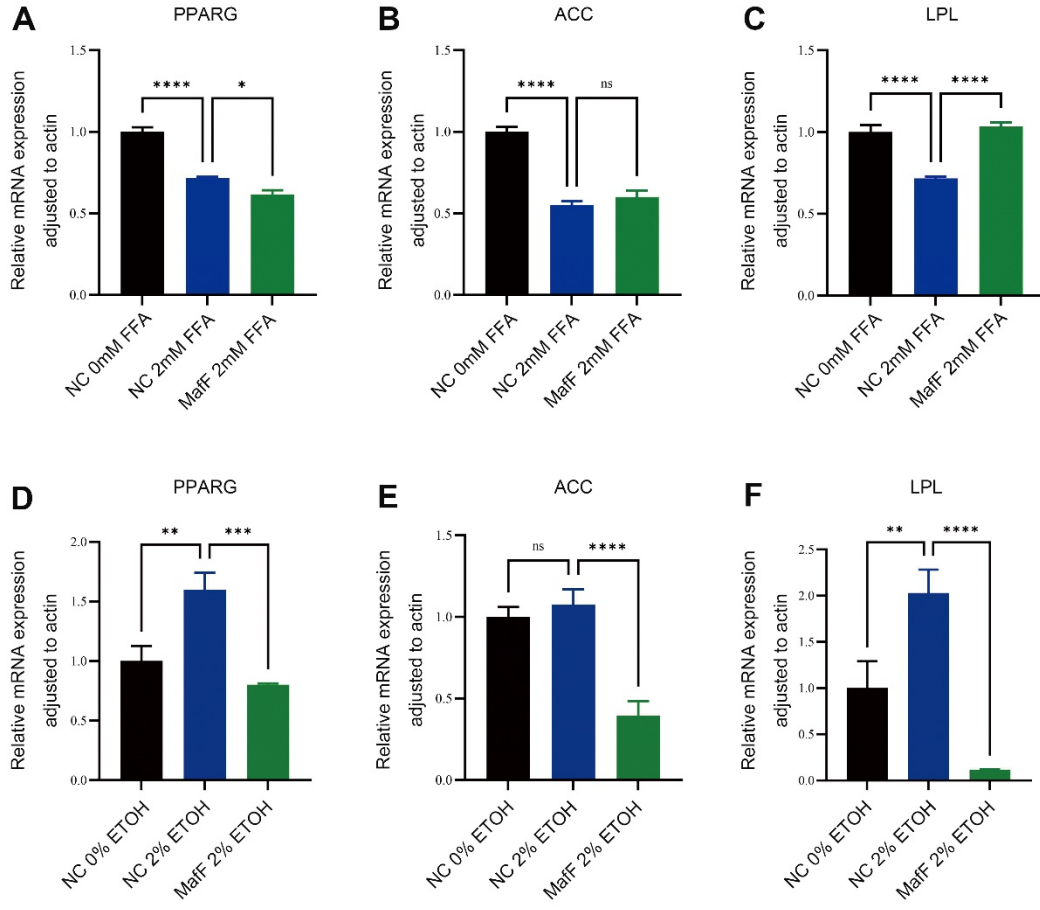


Figure 5



Abc