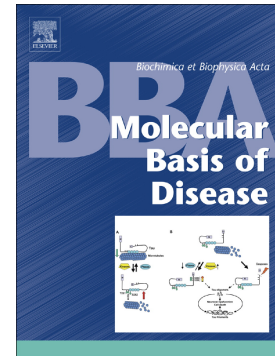


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Inhibition of EGFR attenuates fibrosis and stellate cell activation in diet-induced model of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. NAFLD begins with steatosis and advances to nonalcoholic steatohepatitis (NASH) and cirrhosis. The molecular mechanisms involved in NAFLD progression are not understood. Based on recent studies showing dysregulation of epidermal growth factor receptor (EGFR) in animal models of liver injury, we sought to determine if inhibition of EGFR mitigates liver fibrosis and HSC activation in NAFLD. We utilized the high fat diet (HFD)-induced murine model of liver injury to study the role of EGFR in NAFLD. The lipid accumulation, oxidative stress, hepatic stellate cell (HSC) activation and matrix deposition were examined in the liver tissues. We also evaluated the EGFR signaling pathway, ROS activation and pro-fibrogenic phenotype in oxidized low density lipoproteins (ox-LDL) challenged cultured HSCs. We demonstrate that EGFR was phosphorylated in liver tissues of HFD murine model of NAFLD. Inhibition of EGFR prevented diet-induced lipid accumulation, oxidative stress, and HSC activation and matrix deposition. In cultured HSCs, we show that ox-LDL caused rapid activation of the EGFR signaling pathway and induce the production of reactive oxygen species. EGFR also mediated HSC activation and promoted a pro-fibrogenic phenotype. In conclusion, our data demonstrate that EGFR plays an important role in NAFLD and is an attractive target for NAFLD therapy.

Keywords: liver fibrosis, EGFR, oxidative stress, stellate cell, NAFLD

Abbreviations

ACC, Acetyl CoA carboxylase; ADAM, A disintegrin and metalloproteinases; SREBP, Sterol regulatory element-binding protein; Adipo, Adiponectin; ALB, Albumin; ALP, Alkaline phosphatase; ALT, Alanine transaminase; AST, Aspartate transaminase; BTC, β -cellulin; COL, Collagen; CTGF, Connective tissue growth factor; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichloro-dihydro-fluorescein diacetate; DEN, Diethylnitrosamine; DHE, Dihydroethidium; ECM, Extracellular matrix; EGFR, Epidermal growth factor receptor; EREG, Eregulin; ERK, Extracellular signal-regulated kinase; FABP, Fatty acid binding protein; HB-EGF, Heparin-binding EGF; HCC, Hepatocellular carcinoma; HFD, High fat diet; HO-1, Heme oxygenase-1; HRP, Horseradish peroxidase; HSC, Hepatic stellate cell; MAPK, Mitogen-activated protein kinase; NAC, N-acetyl cysteine; NAFLD, Non-alcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; NOX, NADPH oxidase; NQO-1, NAD(P)H quinone dehydrogenase 1; Nrf-2, Nuclear factor (erythroid-derived 2)-like 2; Ox-LDL, Oxidized low-density lipoprotein; PDGFR, Platelet-derived growth factor receptor; PI3K, Phosphatidylinositol 3'-kinase; ROS, Reactive oxygen species; α -SMA, Alpha-smooth muscle actin; TC, Total cholesterol; TG, Triglycerides; TGF- α , Transforming growth factor- α ; TGF- β , Transforming growth factor- β ; VEGFR, Vascular endothelial growth factor receptor

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver diseases worldwide [1]. NAFLD, defined as excessive lipid accumulation in the liver, ranges in severity from benign fatty liver to nonalcoholic steatohepatitis (NASH). NASH is characterized by oxidative stress, lipid peroxidation, fibrosis, and inflammation. NASH can progress to cirrhosis, the formation of pre-neoplastic nodules, and the development of hepatocellular carcinoma (HCC) [2]. Day and James (1998) have proposed a two-hit model of NAFLD pathogenesis[3]. The first hit is deposition of free fatty acids and triglycerides in hepatocytes (steatosis), and the second hit is the progression of steatosis to NASH. Both of these hits are accompanied by hepatic lipogenesis and increased oxidative stress and inflammation. Liver fibrosis occurs in most types of chronic liver diseases and involves excessive deposition of extracellular matrix (ECM) proteins [4]. Hepatic stellate cells (HSCs) are thought to play a central role in liver fibrosis [5, 6]. In response to injury and inflammatory stimuli, HSCs lose vitamin A-storing droplets and transdifferentiate into myofibroblasts with induction of α -smooth muscle actin [7]. Activated HSCs are highly proliferative and secrete fibrogenic mediators leading to excessive extracellular matrix deposition [8, 9]. Therefore, inhibiting HSC activation is a prime target for therapeutic intervention.

Many receptor tyrosine kinases are expressed in activated HSCs and are associated with the development of liver fibrosis. These include platelet-derived growth factor receptor (PDGFR) [10], vascular endothelial growth factor receptor (VEGFR) [11], and epidermal growth factor receptor (EGFR) [12]. Only recently, EGFR has been studied in models of liver injury including the diethylnitrosamine (DEN) and the carbon tetrachloride (CCl₄) model [12]. EGFR is composed of a ligand-binding extracellular domain and a tyrosine kinase intracellular domain [13, 14]. EGFR ligands include epidermal growth factor (EGF), transforming growth factor- α (TGF- α), amphiregulin (AR), epiregulin (EREG), β -cellulin (BTC), heparin-binding EGF (HB-EGF), and epigen (EPGN) [15]. Upon binding to a ligand, EGFR forms homo- or heterodimers with other EGFR family members. Following activation of the intrinsic kinase domain, several proteins containing Src-homology 2 domains bind and

activate downstream signaling cascades [16-18]. The main activated downstream signaling pathways are extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K)-Akt pathway [17-19].

EGFR has been well demonstrated as a therapeutic target for cancer treatment and several EGFR inhibitors have been used in clinical as anti-cancer agents. Recently, erlotinib, a small-molecule EGFR inhibitor, has also been shown to reduce the number of activated HSCs by depressing EGFR phosphorylation [12], and is also being evaluated in an ongoing clinical trial (NCT02273362) for fibrogenesis inhibition and HCC prevention. Although EGFR inhibition has been reported to reduce HSCs, the underlying mechanisms are not known. In addition, the role of EGFR signaling has not been elucidated in a more clinically applicable diet-induced model of NAFLD. In this study, we utilized the high fat diet-induced model of liver injury and show that inhibition of EGFR prevents fat accumulation and liver fibrosis. We found the mechanism to involve modulation of oxidative stress. Furthermore, we show that EGFR regulates HSC activation and deposition of matrix proteins through inducing oxidative stress.

2. Materials and methods

2.1 Reagents, cell culture and treatments

Selective EGFR inhibitor tyrphostin AG-1478 and reactive oxygen species scavenger N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Compound 451 was prepared in our laboratory and purified using HPLC with a purity of 99.1% as reported previously [20]. AG1478 (AG) and compound 451 were dissolved in DMSO for *in vitro* experiments and in 0.5% sodium carboxymethyl cellulose (CMC-Na) for *in vivo* studies. Oxidized-low density lipoproteins (ox-LDL) were obtained from Biomedical Technologies (Stoughton, MA). Antibodies against EGFR, p-EGFR, Akt, p-Akt, ERK, p-ERK, GAPDH, transforming growth factor- β (TGF- β), collagen 4, collagen 1, and α -smooth muscle actin (α -SMA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), NAD(P)H quinone dehydrogenase 1 (NQO-1), and heme oxygenase-1 (HO-1) were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz.

An immortalized rat hepatic stellate cell (HSC) line, HSC-T6[21, 22], was obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Gibco, Eggenstein, Germany) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, at 37°C in a humidified incubator under a 5% CO₂ atmosphere.

2.2 Animal studies

Male C57/BL6 mice (8-12 weeks old, weighing 18-22 g) were obtained from the Animal Center of Wenzhou Medical University (Zhejiang, China). All animal care and experimental procedures complied with the ‘Ordinance in Experimental Animal Management’ (Order NO. 1998-02, Ministry of Science and Technology, China), and were approved by the Wenzhou Medical College Animal Policy and Welfare Committee. After an acclimatization period of one week, mice were randomly divided into two weight-matched groups. Mice were fed a standard rodent chow (Ctrl; n=7) or a high-fat diet (HFD, n=28) for 20 weeks. Normal control diet was purchased from Mediscience Diets Co. LTD, Yangzhou, China, containing 10 kcal.% fat, 20 kcal.% protein and 70 kcal.% carbohydrate (Cat. #MD12031); HFD was purchased from the same company (Cat. #MD12033) containing 60 kcal.% fat, 20 kcal.% protein and 20 kcal.% carbohydrate. The detailed composition of these two diets were shown in the supplementary Table S2. At 13th week of feeding, HFD-fed mice were further divided into four groups: vehicle-treated HFD group (n=7, CMC-Na used as vehicle), HFD mice treated with 20 mg/kg AG1478 (AG, n=7), HFD mice treated with 10 mg/kg 451 (451-L, n=7), and HFD mice treated with 20 mg/kg 451 (451-H, n=7). Treatments with compound were carried out by oral gavage once every two days from the 13th week to the 20th week. At the end of the experimental period, the mice were sacrificed by overdose of sodium pentobarbital, and blood and liver tissues were collected. Livers were fixed in 4% paraformaldehyde solution, embedded in paraffin, and sectioned at 5 µm. After dehydration, sections were stained with hematoxylin and eosin (H&E) and masson’s trichrome following

routine techniques. To visualize lipids, frozen sections were stained using Oil Red O. The stained sections were then viewed under a bright field microscope (Nikon, Japan).

2.3 Western blot analysis

Cell and tissue lysate were prepared and protein levels were measured by Bradford assay (Bio-Rad, Hercules, CA). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with 5% milk in tris-buffered saline containing 0.05% Tween-20 for 1.5 h at room temperature. Primary antibody incubations were carried out overnight at 4 °C. HRP-conjugated secondary antibodies (1:5000) and enhanced chemiluminescence reagents (Bio-Rad) were used for detection.

2.4 Reverse transcription and real-time quantitative PCR

Total RNA was isolated from cells and tissues using TRIZOL (Thermo Fisher). Reverse transcription and quantitative PCR were performed using M-MLV Platinum RT-qPCR Kit (Thermo Fisher) in Eppendorf Realplex 4 (Eppendorf, Hamburg, Germany). Primers were obtained from Thermo Fisher. Sequences for primers are presented in Supplementary Table S2). The relative amount of each gene was normalized to the amount of β -actin.

2.5 Determination of ROS generation by fluorescent microscope and flow cytometry

Intracellular ROS levels were measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA). Following treatment of cells with ox-LDL and EGFR inhibitors, cells were incubated with DCFH-DA. ROS was measured through DCF fluorescence using flow cytometry (FACSCalibur, BD Biosciences).

2.6 Determination of liver superoxide production

Liver superoxide production was determined by *in situ* dihydroethidium (DHE) staining. Liver tissues were excised, embedded in OCT compound, and cut into 5 μ m-thick sections. Tissue sections were then incubated with DHE in PBS (10 mmol/L) in a dark and humidified

container at 37°C for 45 min. DHE is oxidized upon reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. The images were viewed using Nikon epi-fluorescence microscope equipped with a digital camera (Nikon, Japan).

2.7 EGFR knockdown in hepatic stellate cells

Six small interference RNA (siRNA) sequences targeting EGFR and non-specific negative control were designed and obtained from Santa Cruz Biotechnology. The sequences for siRNAs are presented in Supplementary Table S3. HSCs were transiently transfected with 1 µg/mL siRNAs using PolyJet transfection reagent (SignaGen Laboratories, USA) in DMEM containing 10% FBS for 48 h. Cell lysates were subjected to Western blot analysis to confirm knockdown. Among six siRNAs, No.6 showed the best efficiency for EGFR knockdown and was selected for further experiments. Transfected cells were treated with ox-LDL after 48 h transfection.

2.8 Determination of NADPH oxidase activity

After cell treatment, NADPH oxidase activity in cells was measured using NADP/NADPH Quantification colorimetric Kit according to the manufacturer's instruction (BioVision Inc., Milpitas, CA). NADPH oxidase-dependent superoxide production was determined in liver tissue lysates using lucigenin chemiluminescence as reported previously.

2.9 Measurement of lipids and markers in blood and liver

Blood samples collected at time of sacrifice were centrifuged to prepare serum. The serum levels of biochemical markers including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), hydroxyproline, and albumin (Alb) were measured using respective commercial kits (Nanjing Jiancheng, Jiangsu, China). Aliquots of liver tissue (50mg) homogenates were extracted with chloroform-methanol according to the procedure of Folch et al[23]. Total cholesterol (TC) and triglycerides (TG) levels in serum and liver were determined using commercial assay kits, respectively (Stanbio Laboratory,

Boerne, TX). The liver TC/TG data were normalized to wet weight of liver. Serum LDL levels were determined using a commercial kit (Nanjing Jiancheng, Jiangsu, China).

2.10 Cell growth assessment

Five thousand HSCs were seeded into each well of 96-well plates. Cells were serum-starved for 24 h and treated with 10 µg/mL ox-LDL for 24 h. For EGFR inhibition studies, cells were pretreatment with 10 µM AG1478 or compound 451 (at 5 or 10 µM). Following treatments, cells were stained with nuclear dye DAPI and counted.

2.11 Statistical analysis

Results are expressed as mean $\pm$ SD of three independent experiments for the *in vitro* studies (each with n = 3), and for *in vivo* experiments (n = 4-7). Statistical significance of differences between groups was obtained by the student's t test or ANOVA multiple comparisons in GraphPad Pro5.0 (GraphPad, San Diego, CA).

3. Results

3.1 EGFR inhibition attenuates high fat diet-induced lipid accumulation in the liver

To investigate the role of EGFR in NAFLD, we utilized the high fat diet (HFD)-induced model of NAFLD [24-27]. We modulated EGFR in this model through two specific small-molecule inhibitors: AG1478 and compound 451. These small molecule inhibitors are potent and specific inhibitors of EGFR signaling by blocking its protein tyrosine kinase activity, and have been effective in host of diseases such as cancer and diabetes [20]. Both of these inhibitors exhibit comparable EGFR-inhibitory activity at both the molecular and cellular levels. B6 mice were maintained on control or high-fat diet for 12 weeks. We then treated mice with EGFR inhibitors AG1478 and compound 451 for an additional 8 weeks. Body weights were higher in HFD-fed mice at week 12 (initiation of treatment period, denoted as 0 in Figure 1A) as expected. Treatment with AG and compound 451 had no effect on body weights and the disparity in weight gain between control and HFD was maintained throughout the treatment period of 8 weeks (Figure 1A). In association with increased weight gain, HFD-fed mice exhibited significantly increased levels of serum and liver triglycerides (TG) and liver total cholesterol (TC) (Figure 1B). Furthermore, serum low density lipoprotein (LDL) levels were also increased by HFD feeding (Figure 1C). Inhibition of EGFR by AG1478 was able to normalize serum triglycerides but essentially had no effect on liver tissue lipids (Figure 1B and 1C). However, treatment with compound 451 significantly decreased liver lipid levels as well as serum TG levels (Figure 1B and 1C). These effects were especially evident at high doses of compound 451 (451H).

We then examined the liver tissues for fat accumulation by Oil Red O staining of frozen sections. As expected, lipid accumulation was markedly increased in the liver tissues of HFD-fed mice compared to the control group (Figure 1D). Liver fat accumulation was associated with increased expression of genes involved in lipogenesis and β -oxidation (Figure 1E). Our results show that relative mRNA levels of acetyl-CoA carboxylase (ACC), sterol-regulatory element binding protein 1c (SREBP 1c), and fatty acid binding protein (FABP) are induced and adiponectin (adipo) are depressed by HFD feeding (Figure 1E). Treatment of HFD-fed mice with AG1478 and 451 for 8 weeks significantly reduced fat

accumulation in the livers (Figure 1D) and restored the mRNA of lipogenic/ β -oxidation genes to levels comparable to control mice (Figure 1E). These results indicate that EGFR inhibition could attenuate the HFD-induced lipid accumulation in the liver.

3.2 *EGFR inhibition prevents liver fibrosis*

We performed H&E staining of liver tissues to evaluate the extent of liver damage (Figure 2A, upper panel). Histological examination showed normal liver architecture in control mice and structural abnormalities and fatty degeneration in the HFD-fed group. Treatment with EGFR inhibitors showed marked improvement in tissue morphology and architecture compared vehicle-treated HFD-fed group (Figure 2A). We then assessed liver fibrosis through Masson's trichrome staining and show increased collagen deposition in the HFD-fed mice (Figure 2A, lower panel). Similar to our observations in H&E stained sections, we found that EGFR inhibition prevented collagen deposition in liver tissues of mice fed a HFD. We confirmed these findings by western blot analysis of collagen 4 and pro-fibrotic transforming growth factor- β (TGF- β) expression (Figure 2B and supplementary Figure S1). Both proteins were elevated in HFD-fed mice and decreased upon EGFR inhibition. More appreciable changes were noted for TGF- β with EGFR inhibition. mRNA levels of both collagen 4 and TGF- β also showed the same pattern (Figure 2C). Lastly, we measured hydroxyproline levels in serum as a proxy for collagen deposition and fibrosis. HFD increased the serum levels of hydroxyproline (Figure 2D). However, for this readout of fibrosis, only compound 451 significantly reduced hydroxyproline levels.

We then assessed the liver function to determine whether structural abnormalities and fibrosis corresponds to functional alterations. Analysis of liver injury and cholestasis markers paralleled structural abnormalities in HFD-fed mice (Figure 1E-H). Improvements were noted for several markers upon EGFR inhibition. These can be seen for alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP). AG1478 and compound 451 also restored the synthetic function as assessed by significant increases in serum albumin (ALB) levels. Overall, compound 451 displayed stronger anti-fibrotic activity compared to AG1478. This can be seen with hydroxyproline levels and

Masson trichrome staining when AG1478 and compound 451 were used at a similar dose of 20 mg/kg.

3.3 HFD activates EGFR signaling pathway and induces oxidative stress in the liver

EGFR has been recently shown be phosphorylated in animal models of liver injury including the DEN mouse model, CCl₄ rat model, and a rat model induced by bile duct ligation (BDL)[12]. As no studies have investigated EGFR activation in diet-induced model of NAFLD, we wanted to assess whether the EGFR signaling proteins are phosphorylated upon HFD feeding. We noted increased phosphorylation of EGFR and downstream proteins including Akt and extracellular signal-regulated kinase (ERK). Both AG1478 and compound 451 at 10 mg/kg (low dose) effectively normalized increased levels of phosphorylated EGFR, Akt, and ERK. Treatment of mice with high dose compound 451 (20 mg/kg; comparable to AG1478) decreased the phospho-proteins to levels lower than controls (Figure 3A and supplementary Figure S2).

To gain an understanding of the mechanisms downstream of EGFR in potentially regulating fibrosis and liver injury, we looked towards reactive oxygen species (ROS) and oxidative stress. Accumulating evidence shows that oxidative stress plays a pivotal role in the progress of liver fibrosis and cirrhosis[28, 29]. Increased indices of oxidative stress have been reported in liver tissues of mice maintained on HFD [8]. Furthermore, EGFR is also implicated in lung [30]and kidney [31]fibrosis through induction of oxidative stress. Thus, we sought to determine whether oxidative stress was involved in liver fibrosis in HFD-fed mice and whether EGFR inhibition modulated this axis. We utilized *in situ* dihydroethidium (DHE) fluorescence staining to examine superoxide anion production in liver tissues (Figure 3B). DHE forms a red fluorescence product which intercalates with DNA upon reaction with superoxide. Our results show higher levels of superoxide in liver tissues of mice fed a HFD (Figure 3B). Both AG1478 and compound 451 effectively blocked superoxide production as evident by complete normalization of DHE staining. Compound 451 at higher doses was comparable to AG1478. These findings indicate that EGFR signaling activation may be involved in the oxidative stress in HFD-induced liver fibrosis.

A potential source of ROS in liver tissues is the NADPH oxidase (NOX) system[32, 33]. Several major isoforms of are expressed in the liver including NOX2 and NOX4[33]. We investigated the involvement of the NOX system by assessing NOX-dependent superoxide production in tissue lysates and analysis of NOX expression. As shown in Figure 3C, NOX-dependent superoxide production was elevated in HFD-fed mice, while AG1478 and compound 451 treated mice showed no such increases. mRNA analysis showed increased levels of both NOX2 and NOX4 in liver tissues of HFD group but not in the EGFR-inhibited HFD group (Figure 3D and 3E). In agreement with these results, anti-oxidant response proteins including nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NAD(P)H dehydrogenase quinone 1 (NQO-1) were depressed in liver tissues of mice fed a HFD (Figure 3F-3I and supplementary Figure S3). These alterations were normalized to a large extent by EGFR inhibition.

The main cell types involved in hepatic fibrogenesis are the hepatic stellate cells (HSCs). HSCs are normally quiescent but undergo activation in response to liver injury and synthesize fibrotic matrix rich in collagen[34]. Although HFD feeding has been shown to activate HSCs which is denoted by increased expression of α -smooth muscle actin (α -SMA)[8, 9], the role of EGFR in this process is not fully known. Based on our findings, we suspected that EGFR may regulate stellate cell activation. Therefore, we measured α -SMA transcript and protein levels in liver tissues of mice fed a HFD and treated with EGFR inhibitors. As shown in Figure 3J-K, HFD-induced α -SMA was normalized by both AG1478 and compound 451 treatments (Figure 3K and supplementary Figure S4). Collectively, our findings show ROS generation, downstream of EGFR, in mediating HFD-induced liver fibrosis and possible activation of HSCs.

3.4 EGFR inhibition inhibited ox-LDL-induced EGFR signaling activation, oxidative stress, HSCs activation

We performed *in vitro* studies using HSCs to dissect the mechanism of EGFR-mediated cell activation and fibrogenic matrix deposition. It has previously been demonstrated that oxidized-LDL (ox-LDL) prompts biosynthesis of matrix components in HSCs[35, 36]. This

same mechanism may be at play in our *in vivo* studies as we have shown increased fat accumulation and oxidative stress. We tested whether ox-LDL in culture would phosphorylate EGFR in HSCs. We treated cells with ox-LDL for different time periods and noted increased EGFR phosphorylation as early as 15 min after stimulation with a peak at 1 h (Figure 4A). EGFR phosphorylation followed levels of p-Akt and -ERK. Pre-treatment of cells with EGFR inhibitors AG1478 and compound 451 completely negated ox-LDL-induced activation of the EGFR pathway (Figure 4B and supplementary Figure S5). We then determined oxidative stress and cell activation status in HSCs. We show that ox-LDL exposure increases intracellular ROS levels as evidenced by increased DCF-DA fluorescence (Figure 4C). Since DCF-based fluorescent results may be affected by some compounds which produce hydrogen peroxide in rapid flux, we set up the 451 alone group and AG1478 alone group to avoid the possible interference produced by compounds 451 and AG1478. As shown in the Figure 4C, the MFI (Mean Fluorescence Intensity) value in control group is similar with that in 451 alone group and AG1478 alone group, indicating AG1478 and 451 did not affect the DCF-based fluorescent assay. However, 451 and AG1478 pretreatment remarkably inhibited ox-LDL-increased MFI value in HSCs. Validating our *in vivo* findings, treatment of cultured HSCs with EGFR inhibitors reduced intracellular ROS levels.

As mentioned above, HSC activation is a key step in liver injury and leads to deposition of fibrogenic matrix. We assessed whether EGFR modulated ox-LDL-induced activation of HSCs by examining the expression α -SMA and cell growth. Figures 4D and 4E show increased expression of α -SMA in response to ox-LDL exposure. Ox-LDL stimulation was also associated with increased HSC numbers in culture (Figure 4F and 4G). This activation was mediated by EGFR as both AG1478 and compound 451 dampened ox-LDL-induced expression of α -SMA and cell numbers (Figures 4D-4G and supplementary Figure S6). Together, findings showing increased ROS levels, cell numbers, and induction of α -SMA clearly point to an activate HSC state. This activated state is also associated with increased expression of collagen 1 and TGF- β at both the mRNA and protein levels (Figure 4H and 4I), implicating EGFR in mediating these changes. Pretreatment of HSCs with AG1478 and

compound 451 attenuated ox-LDL-induced fibrogenic programming (Figure 4H and supplementary Figure S7).

3.5 EGFR silencing prevents ox-LDL-induced ROS generation and HSCs activation

To provide conclusive evidence for the role of EGFR in mediating ox-LDL-induced oxidative stress and HSC activation, we knocked down the expression of EGFR using siRNA. Six siRNA sequences for EGFR were designed, and among them, the No.6 siRNA showed the best efficiency for EGFR knockdown (supplementary Figure S8 and Figure 5A). HSCs were transfected with control sequence or No. 6 siRNA sequence and then were treated with ox-LDL. Creating EGFR deficit led to complete prevention of ox-LDL-induced expression of collagen 1, TGF- β , and α -SMA (Figures 5B-5E and supplementary Figure S9). These results show that EGFR is involved in ox-LDL-induced HSC activation and expression of fibrogenic matrix. Associated with normalized HSC phenotype, EGFR silencing also prevented the ox-LDL-induced ROS production as detected by DCF fluorescence (Figure 5F). Reduced ROS levels seen in HSCs may be involved in HSC activation and promotion of matrix synthesis. Using the same platform, we pretreated HSCs expressing control or EGFR siRNA with a ROS scavenger, N-acetyl cysteine (NAC). We have previously used NAC to elucidate the role of ROS in targeting cancer cells[37-39]. Here we show that pretreatment of HSCs with NAC (10 mM) decreased the expression of collagen 1, TGF- β , CTGF, and α -SMA induced by ox-LDL stimulation (Figure 6A, 6B and supplementary Figure S10). These findings support the notion that ox-LDL increases ROS production in HSCs leading to cell activation and deposition of matrix.

4. Discussion

NAFLD is characterized by remodeling of the ECM and excessive deposition of collagen. Important cellular mediators of these changes are the HSCs which transdifferentiate into myofibroblasts and deposit fibrogenic matrix. In our study utilizing a HFD-fed mouse model of NALFD, we show significant lipid accumulation in liver tissues in association with EGFR pathway activity. Treatment of mice with two specific inhibitors of EGFR, AG1478 and

compound 451, attenuated lipid accumulation without significant changes to serum LDL levels. Liver tissues of HFD-fed mice also showed increased ROS production possibly through dysregulated NADPH oxidase system. Using this context, we modeled HSC activation in culture through exposure to ox-LDL and assessed the involvement of EGFR. Our studies demonstrate that ox-LDL activates HSCs and produces a pro-fibrogenic phenotype as evidenced by increased matrix and fibrogenic growth factor expression. These activities were mediated through EGFR pathway activation and involved generation of ROS. We were able to mitigate these activities by inhibiting EGFR as well as by knocking down its expression. Furthermore, we show that removing ROS from the equation prevents ox-LDL induced HSC activation. Our studies suggest that EGFR plays a detrimental role in the pathogenesis of HFD-induced liver damage and that exacerbated EGFR phosphorylation regulates lipid accumulation and liver fibrosis, likely through increased liver oxidative stress.

HSC activation is well established to play a role in the development and progression of liver fibrosis. These peculiar cells are defined as fat-storing pericytes that constitute 5-8% of all liver cells [40]. HSCs are quiescent but are activated through inflammatory stimuli and through paracrine interactions with Kupffer cells to become highly proliferative. Associated with this proliferative phase is the ability to synthesize and deposit matrix proteins. In experimental models of liver injury, this activated phase is denoted by induction of HSCs α -SMA [8, 9]. Our studies show that HFD-feeding induces α -SMA in the liver tissues. We further show co-induction of collagen and fibrogenic TGF- β . Both of these readouts of HSCs are prevented when mice are treated with EGFR inhibitors. In addition, ox-LDL exposure of HSCs in culture improves growth and mimics activation as illustrated through α -SMA induction and matrix deposition.

The precise mechanism by which EGFR activation facilitates HSC transdifferentiation is not known. However, we are able to speculate that the mechanism may involve ROS production. ROS may be derived from HSCs themselves or from the environment. Our studies show that ROS is induced when HSCs are exposed to ox-LDL suggesting an autocrine source. ROS may also be produced by Kupffer cells [41] and damaged hepatocytes [42]. In both cases, however, it is expected that a pro-inflammatory and imbalanced redox

environment is created. Agents which display anti-oxidative effects protect the liver from injury induced by hepatotoxins and prevent fibrogenesis [43, 44]. In the present study, we found that oxidative stress is remarkably increased in the livers of HFD-fed mice in association with liver fibrosis and injury. In addition, we show that ROS scavenging by NAC prevent HSC activation and fibrosis.

An interesting focus of future studies may be delineating how EGFR is activated in NAFLD. A simple mechanism may involve increased expression and availability of EGFR ligands. EGFR ligands are synthesized as transmembrane precursors and are proteolytically cleaved [19] by ADAMs (a disintegrin and metalloproteinases) [45-47]. In a model of Fas-mediated liver injury, EGFR ligands AR, EREG and TGF- α were all shown to be upregulated [48]. However, in this case, EGFR activation may be protective as administration of AR prevents Fas-mediated liver injury and shows direct anti-apoptotic effects in primary hepatocytes. TGF- α overexpression is also protective in Fas-mediated hepatic apoptosis [49, 50]. Furthermore, loss of ADAM17 (the main ADAM involved in cleavage of EGFR ligands) in hepatocytes promotes Fas-induced apoptosis [51]. All of these studies suggest that EGFR activity may be hepatoprotective. Therefore, if overexpression and availability is the mechanism in NAFLD, then the context becomes important in mediating detrimental effects of EGFR activation.

A more likely mechanism of EGFR involvement in NAFLD may be transactivation. It is possible that involvement of atypical signaling proteins downstream of EGFR through transactivation mediate the adverse effects. We know that EGFR is able to form complexes with other receptor tyrosine kinases to initiate signaling, including PDGFR [52, 53] and insulin-like growth factor 1 receptor [54]. This hetero-receptor complex may form by both a ligand-dependent and a ligand-independent mechanism [52, 54]. Furthermore, EGFR may be transactivated by several other factors including interferon- γ - and growth hormone-bound receptors [55, 56], integrins [57], and g-protein-coupled receptors (GPCRs) [45, 46, 58-60]. Although, deciphering this complicated signaling pathway and its involvement in NAFLD is daunting, it is also exciting.

5. Conclusions

EGFR inhibitors already constitute the first-line therapy for a number of cancers and our studies suggest another clinically significant indication where EGFR inhibitors may be of therapeutic benefit. We have provided evidence for a detrimental role of EGFR in the pathogenesis of HFD-induced liver damage. However, the approach using shRNA knockdown or gene knockout in vivo with HFD feeding would be more robust to support our conclusion. This is a limitation of this study. Further, the role of EGFR has to be evaluated via use of appropriate transgenic mice or shRNA-based knockdown approaches in vivo. We also show that the mechanism may involve elaboration of ROS and oxidative stress in the liver tissue. Finally, our studies show that the newly identified EGFR inhibitor compound 451 has comparable effects in improving the liver fibrosis in HFD mice as AG1478. Further studies assessing the clinical utility of compound 451 are warranted.

Conflict of interest statement

All the authors declare no competing financial interest.

Author contributions

D.L., H.C., W.Z., J.H., Z.L., and P.Z. performed the research; G.L. and L.Z. designed the research study; J.W. and W.W. contributed essential reagents or tools; G.L., D.L., L.Z., and J.W. analysed the data; G.L. and D.L. wrote the paper.

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Figure Legends

Figure 1. Inhibiting EGFR prevents diet-induced fat accumulation in the liver.

A. Body weight of mice maintained on control or high-fat diet (HFD) for 8 weeks [x-axis = weeks; HFD+AG = HFD mice treated with 20 mg/kg AG1478, HFD+451L = HFD mice treated with 10 mg/kg compound 451; HFD+451H = HFD mice treated with 20 mg/kg compound 451; untreated HFD mice received CMC-Na vehicle; n = 7]. **B.** Serum and liver tissue levels of total cholesterol (TC) and triglycerides (TG) presented in mg/dL. **C.** Serum low-density lipoproteins (LDL) levels in mice treated as in panel A. **D.** Oil Red O staining of frozen liver tissues from mice maintained on control diet or HFD. HFD mice were treated with EGFR inhibitors AG1478 and compound 451 for 8 weeks [red = neutral lipid droplets; blue = hematoxylin]. **E.** Real-time qPCR assay showed the mRNA levels of markers of adipocytes and genes involved in fatty acid metabolism [mRNA levels normalized to β -actin; ACC = acetyl-CoA carboxylase; SREBP = sterol-regulatory element binding protein; FABP = fatty acid binding protein; Adipo = adiponectin. [n = 7; * p<0.05 and ** p<0.01 versus HFD; # p<0.05, ## p<0.01, and ### p<0.001 versus vehicle control (Ctrl)]

Figure 2. AG1478 and compound 451 attenuate high fat diet-induced fibrosis in the liver.

A. Histological analysis of liver tissues showing H&E (upper panel) and Masson's trichrome staining (lower panel; collagen = blue, cytoplasm = red). **B.** Representative western blot analysis of collagen 4 (Col 4) and transforming growth factor- β (TGF- β ; isoform 1). GAPDH was used as loading control. The densitometric quantification results were shown in the supplementary Figure S1. **C.** Real-time qPCR assay showed the mRNA levels of collagen 4 (alpha chain) and TGF- β in liver tissues of mice [mRNA levels normalized to β -actin]. **D.** Serum hydroxyproline level as readout of collagen deposition. **E-H.** Liver function tests showing the serum level of alanine transaminase (ALT, E), aspartate transaminase (AST, F), alkaline phosphatase (ALP, G), and albumin (ALB, H). [n = 7; * p<0.05 and ** p<0.01 versus HFD; # p<0.05 and ## p<0.01 versus vehicle control (Ctrl)]

Figure 3. High fat diet activates EGFR signaling pathway and induces oxidative stress.

A. Analysis of proteins in the epidermal growth factor receptor (EGFR) signaling pathway in liver tissues of mice. Figure showing EGFR, Akt, ERK, and corresponding phosphorylated forms. **B.** Micrographs of dihydroethidium (DHE)-stained liver tissues showing superoxide production [red = oxidized form]. **C.** NADPH oxidase-dependent superoxide production in liver tissue lysates as assessed by lucigenin chemiluminescence. Superoxide production is reported as relative light units (RLU)/ min/ mg protein. **D-E.** Real-time qPCR assay showed the mRNA levels of NADPH oxidase-2 (NOX-2, D) and -4 (NOX-4, E) in liver tissues of mice maintained on control diet and HFD. The mRNA levels normalized to β -actin. **F.** Protein analysis showing levels of Nrf-2, NQO-1, and HO-1. **G-I.** Real-time qPCR assay showed the mRNA levels of Nrf-2 (G), NQO-1 (H), and HO-1 (I) in liver tissues [mRNA levels normalized to β -actin]. **J-K.** mRNA (J) and protein (K) expression of α -SMA in liver tissues [mRNA levels normalized to β -actin]. For real time-qPCR assay, n = 7 mice in each group; * p<0.05 and ** p<0.01 versus HFD; ^{##} p<0.01 and ^{###} p<0.001 versus Ctrl. For panels A, F, and K, representative blots were shown and the densitometric quantification results were shown in the supplementary Figure S2-S4, respectively.

Figure 4. Oxidized LDL induces EGFR activation, oxidative stress, and hepatic stellate cell activation.

A. Activation of EGFR signaling pathway showing levels of phosphorylated EGFR, Akt, and ERK in HSCs treated with 10 μ g/mL ox-LDL for indicated time periods. **B.** Effect of EGFR inhibitors on ox-LDL induced EGFR signaling pathway. Figure showing phosphorylated EGFR, Akt, and ERK in cells treated with 10 μ g/mL ox-LDL in the presence of AG1478 and compound 451. Treatments were carried out for 1 h [AG = 10 μ M AG1478, 451(5) = compound 451 at 5 μ M, 451(10) = compound 451 at 10 μ M]. **C.** Intracellular ROS levels in HSCs treated with EGFR inhibitors. Cells were treated as in panel B (adding 451 alone

group and AG1478 alone group) and stained with DCFH-DA. ROS levels were determined by measuring DCF fluorescence [values in the figure legend show mean fluorescence intensity]. **D-E.** Protein (D) and mRNA (E) levels of α -SMA in HSCs treated with ox-LDL with or without EGFR inhibitors (mRNA levels were measured by real-time qPCR assay and normalized to β -actin). **F-G.** Total live cell numbers following treatment of HSCs with ox-LDL and EGFR inhibitors. Cells were treated and stained with nuclear dye DAPI (F) and total cells were calculated for panel G (normalized to control and presented as % control). **H.** Measures of fibrosis showing Collagen 1 and TGF- β levels in HSCs treated with ox-LDL and EGFR inhibitors. **I.** Real-time qPCR assay showed the mRNA levels of collagen 1, TGF- β , and CTGF in HSCs [mRNA levels normalized to β -actin]. For panels B, D, and H, representative blots were shown among 3 independent experiments, and the densitometric quantification results were shown in the supplementary Figure S5-S7, respectively. For panels E, G, and I, n= 4 independent experiment (* p<0.05 and ** p<0.01 versus ox-LDL; # p<0.05 and ## p<0.01 versus Ctrl).

Figure 5. Knockdown of EGFR prevents ox-LDL induced fibrogenic protein expression in stellate cells.

A. Knockdown of EGFR expression by siRNA in HSCs. Assessment of EGFR protein levels was performed 24h following transfection. **B.** Western blot analysis of fibrogenic proteins following treatment of control- and EGFR siRNA expressing cells with 10 μ g/mL ox-LDL for 1 h. Representative blots were shown among 3 independent experiments, and the densitometric quantification result was shown in the supplementary Figure S9, respectively. **C-E.** Real-time qPCR assay showed the mRNA levels of collagen 1 (C), TGF- β (D), and α -SMA (E) in control or EGFR siRNA transfected HSCs treated with ox-LDL. Treatment with ox-LDL was carried out for 1h [mRNA levels normalized to β -actin (n= 4 independent experiments; * p<0.05 and ** p<0.01 versus ox-LDL; ## p<0.01 versus Ctrl). **F.** Intracellular ROS levels in HSCs as assessed by DCF fluorescence. Cells were treated as indicated for panel B above. Following treatment, cells were incubated with DCFH-DA and ROS-mediated DFC levels were measured by flow cytometry (values in the figure legend

show mean fluorescence intensity).

Figure 6. Oxidative stress mediates fibrosis and stellate cell activation.

A. HSCs were exposed to 10 μ g/mL ox-LDL for 1 h with or without a 1 h pretreatment with 5 μ M N-acetyl cysteine (NAC). Real-time qPCR assay showed the mRNA levels of fibrogenic genes: collagen 1, TGF- β , CTGF, and α -SMA (mRNA levels normalized to β -actin; n=4, * p<0.05, ** p<0.01, and ***p<0.001 versus ox-LDL; # p<0.05, ## p<0.01, and ### p<0.001 versus Ctrl). **B.** Protein levels of collagen 1, TGF- β , and α -SMA as determined by western blot analysis. Cells were treated as in panel A. Representative blots were shown among 3 independent experiments, and the densitometric quantification results were shown in the supplementary Figure S10.

Figure 1

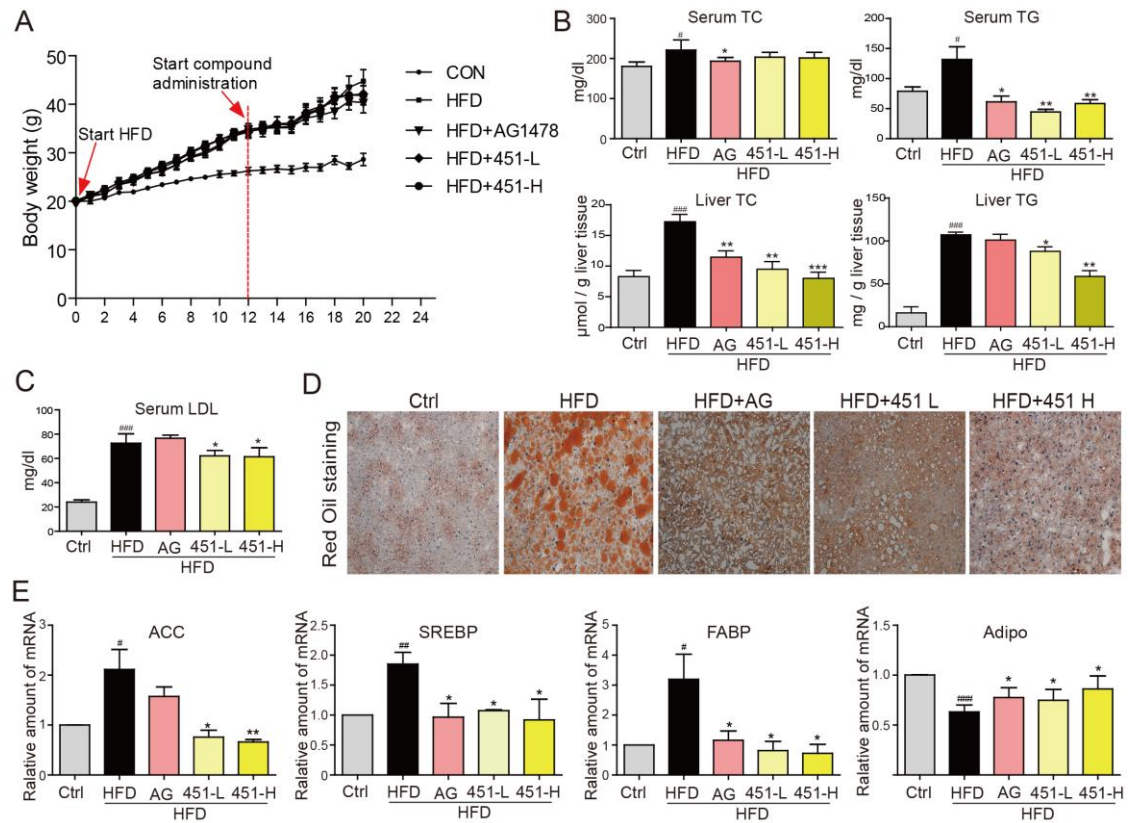


Figure 2

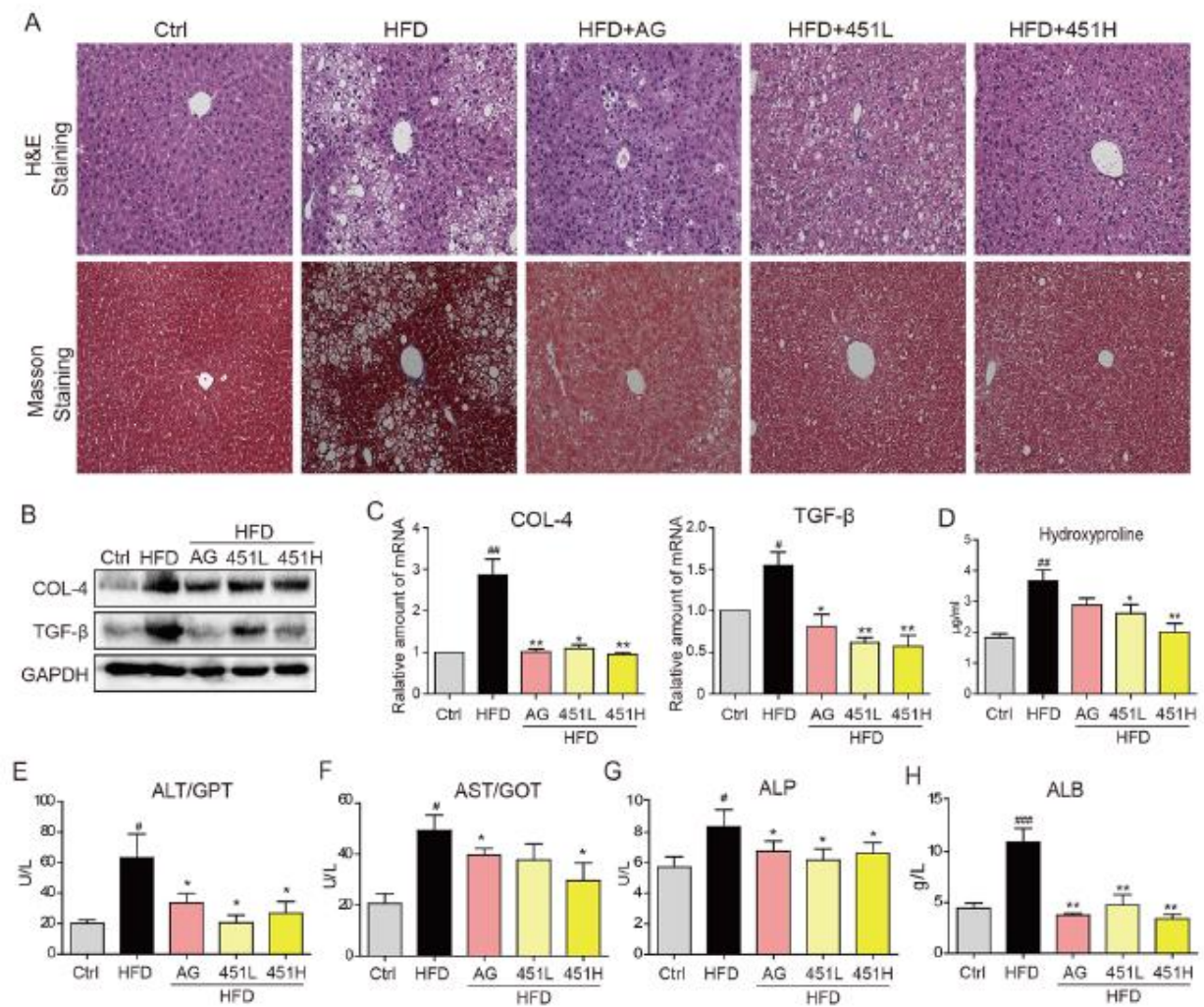


Figure 3

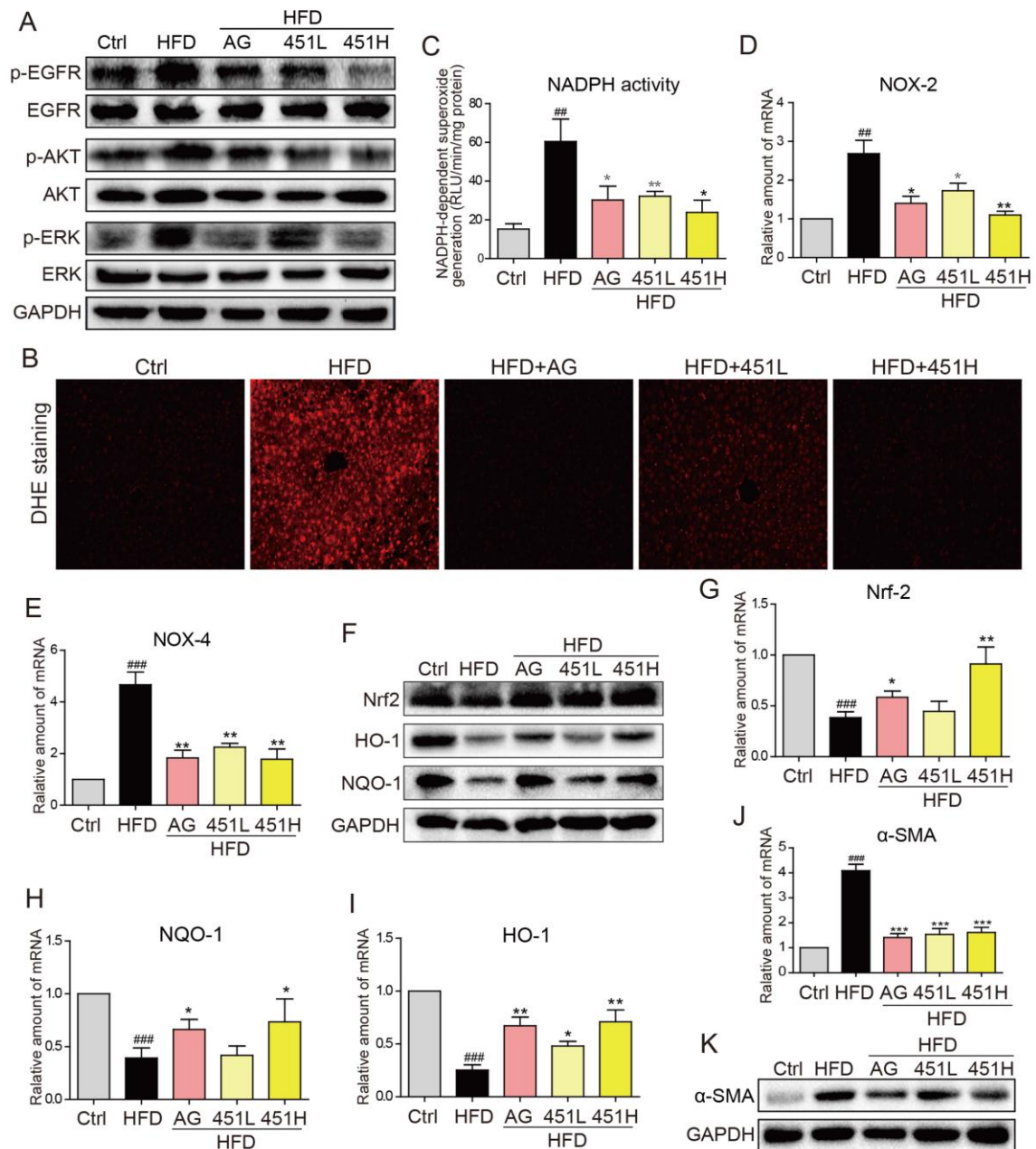


Figure 4

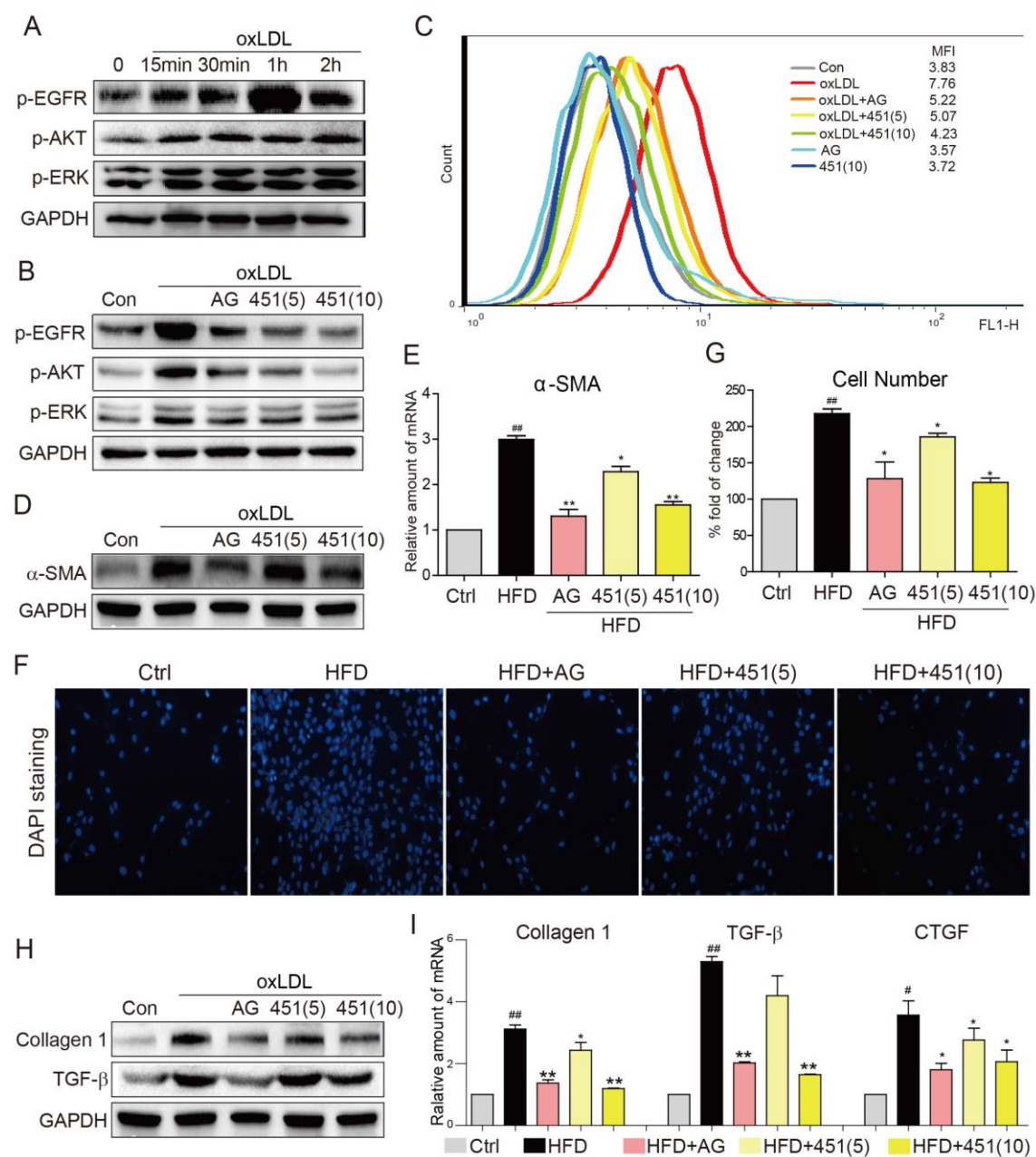


Figure 5

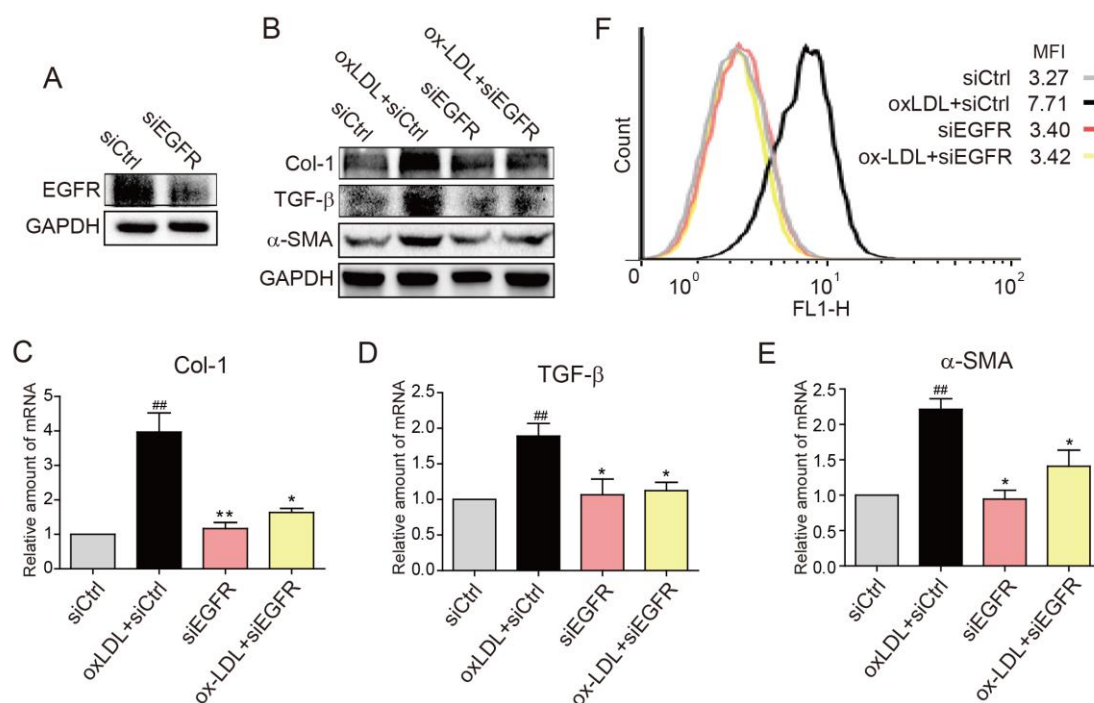
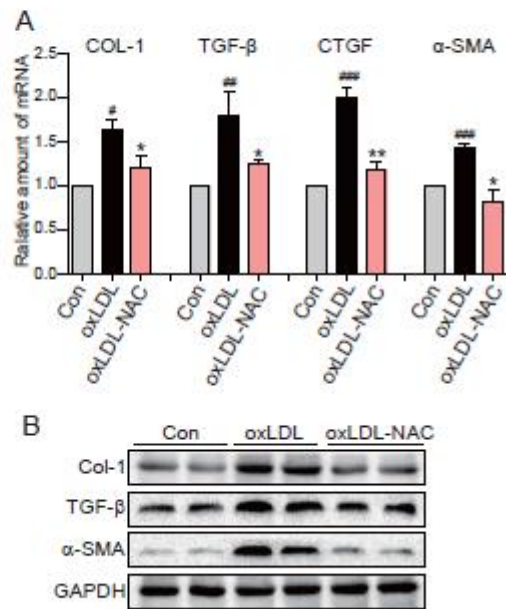


Figure 6



Highlights:

1. EGFR plays a detrimental role in the pathogenesis of HFD-induced liver damage.
2. EGFR mediates HFD-induced liver damage via elaboration of ROS and oxidative stress.
3. Inhibition of EGFR prevented HSC activation.
4. EGFR inhibitor can prevent the liver fibrosis in HFD mice.