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ARTICLE

Liver X receptor agonists exert antitumor effects against hepatocellular carcinoma via inducing REPS2 expression

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Recent studies show that liver X receptor (LXR) agonists exert significant antitumor effects in a variety of tumor cell lines including hepatocellular carcinoma (HCC). But the molecular mechanisms underlying LXR antitumor activity are not fully understood. In this study we investigated the effect of LXR agonist T0901317 (T317) on HCC development and its relationship with RalA binding protein 1 (RALBP1)-associated EPS domain containing 2 (REPS2)/epidermal growth factor receptor (EGFR) signaling axis. We showed that T317 (0.1−0.5 μM) dose-dependently increased REPS2 expression in normal hepatocytes (BNLCL.2 and LO2) and HCC cells (HepG2 and Huh-7). Using promoter activity assay and chromatin immunoprecipitation (CHIP) assay we demonstrated that T317 enhanced REPS2 expression at the transcriptional level via promoting the binding of LXR protein to the LXR-response element (LXRE) in the REPS2 promoter region. We showed that the inhibitory effect of T317 on the proliferation and migration of HCC cells was closely related to REPS2. Moreover, we revealed that T317 (400 nM) increased expression of REPS2 in HepG2 cells, thus inhibiting epidermal growth factor (EGF)-mediated endocytosis of EGFR as well as the downstream activation of AKT/NF-κB, p38MAPK, and ERK1/2 signaling pathways. Clinical data analysis revealed that REPS2 expression levels were inversely correlated with the development of HCC and reduced REPS2 expression associated with poor prognosis, suggesting that REPS2 might be involved in the development of HCC. In conclusion, this study provides new insights into the potential mechanisms of LXR agonist-inhibited HCC.

Keywords: HCC; LXR; REPS2; EGFR; endocytosis; T0901317

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INTRODUCTION

Liver X receptors (LXRs), members of the nuclear transcription factor superfamily, have two isoforms, LXRα and LXRβ [1]. LXRα is predominantly expressed in metabolically exuberant tissues such as liver, adipose, and macrophages, while LXRβ is ubiquitously expressed throughout the body, and the proteins encoded by these genes are highly concordant between species [2]. In vivo, hydroxylated cholesterols can serve as endogenous ligands for LXR to activate its transcriptional activity [e.g., 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, 24(*S*),25-epoxycholesterol] [3]. T0901317 (T317) is a synthetic exogenous small molecule agonist for LXRs. In the nucleus, the DNA-binding domain of LXR recognizes specific sequences known as LXR-response element (LXRE) with the consensus sequence AGGTCAn_4AGGTCA. Under the stimulation of endogenous or exogenous LXR ligands, LXR can bind with retinoid x receptor (RXR) to form a heterodimeric complex that binds to LXRE to promote target gene transcription [4].

LXRs are known as cholesterol sensors and play a central role in the regulation of lipid metabolism. LXRs are activated in many kinds of cells in response to increased cholesterol levels, thereby promoting the expression of cholesterol reverse transporters, such as ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) [5, 6], on the cell membrane to transport intracellular cholesterol out of the cell. In addition, studies have shown that LXR can activate UDP-glucuronosyltransferase 1A3 (UGT1A3) and cholesterol 7-alpha hydroxylase (CYP7A1) to promote bile acid catabolism and maintain bile acid homeostasis [7]. Activation of ATP-binding cassette subfamily G member 5 (ABCG5) and ATP-binding cassette subfamily G member 8 (ABCG8) by LXR promotes free cholesterol entry into the gallbladder in the liver, thereby playing an important role in bile acid synthesis, metabolism, and secretion [8]. In addition to their function in lipid metabolism, LXRs have also been found to regulate immune and inflammatory responses. LXRs play an important role in regulating cytokine production and anti-inflammatory responses [9, 10]. LXR activation has been demonstrated to have antitumor effects in breast cancer, colorectal cancer, and others, suggesting that LXR is a potential target in cancer prevention and therapy [11–13]. The tumor LXR level is a prognostic marker for patients with HCC and significantly inhibit

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the development of HCC [14, 15]. However, the underlying mechanisms are not fully understood.

RalA binding protein 1 (RALBP1)-associated EPS domain containing 2 (REPS2) was identified in the 1990s, and the protein encoded by this gene is involved in the endocytosis of growth factor receptors [16]. REPS2 can bind to RALBP1, thereby inhibiting the RALBP1/RAL signaling pathway to inhibit the endocytosis of epidermal growth factor (EGF) and insulin [17, 18]. REPS2 is also involved in the progression of prostate cancer. Downregulation of REPS2 expression was concurrent with the androgen-dependent to androgen-independent transition of prostate cancer [19]. Mechanistic studies have shown that REPS2 binds to p65 reciprocally to inhibit the activity of NF-κB [20]. REPS2 is also involved in the development and prognosis of breast cancer, esophageal squamous cell carcinoma, colorectal cancer, etc [21–23]. However, the role and possible molecular mechanism of REPS2 in HCC are not clear.

Epidermal growth factor receptor (EGFR) and its ligand EGF are essential for cell proliferation, differentiation, and tumorigenesis [24, 25]. Notably, EGFR signaling is frequently activated in HCC to promote hepatocarcinogenesis and progression, and is associated with an aggressive phenotype, intrahepatic metastasis and poor clinical outcome [26, 27].

The LXR agonist T317 was shown to limit the growth of primary HCC by restricting transforming growth factor beta 1 (TGF-β)-dependent cancer-associated fibroblast (CAF) differentiation [15]. T317 also inhibits HCC progression by inducing of LXRα while reducting of glucose transporter type 1 (Glut1) and matrix metallopeptidase 9 (MMP9) expression and glucose content in HCC cells [28]. However, additional mechanisms underlying the tumor inhibitory activity of T317 may also exist. In the current study, we found that the expression of REPS2 was significantly decreased in LXRα/β knockdown HepG2 cells, and a potential LXRE was also found in the promoter region of REPS2. Therefore, we speculate that LXR activation may directly induce REPS2 expression, which further affects the EGFR signaling pathway to inhibit the proliferation and migration of HCC cells.

MATERIALS AND METHODS

Reagents

T317 was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-EGFR, phospho-EGFR (p-EGFR), β-actin and GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, UAS). Rabbit anti-REPS2, NF-κB and phospho-NF-κB (p-NF-κB) polyclonal antibodies were purchased from Affinity Biosciences (Cincinnati, OH, USA). Rabbit anti-LXRα, LXRβ, ERK1/2, and phospho-ERK1/2 (p-ERK1/2) and mouse anti-AKT and phospho-AKT (p-AKT) polyclonal antibodies were purchased from Proteintech (Chicago, IL, USA). Rabbit anti-p38MAPK and phospho-p38MAPK (p-p38MAPK) polyclonal antibodies were purchased from ABclonal Technology (Woburn, MA, USA). Human EGF was purchased from Sino Biological (Littleton, CO, USA). The dual-luciferase assay kit was purchased from Promega (Madison, WI, USA). LY294002 (PI3K inhibitor), U0126 (MEK1/2 inhibitor) and SB203580 (p38MAPK inhibitor) were purchased from LC Laboratories (Woburn, MA, USA).

Cell culture

HepG2, Huh-7 and LO2 cells, human hepatic cell lines, and BNLCL.2 cells, a murine hepatic cell line, were purchased from ATCC (Manassas, VA) and cultured in complete RPMI-1640 or DMEM medium containing 10% FBS, 50 μg/mL penicillin/streptomycin, and 2 mM glutamine, respectively. LXRα/β knockout cell lines were generated by the CRISPR-Cas9 technique with HepG2 cells as described previously [29]. Briefly, the guide sequence was designed based on the online CRISPR Design Tool (http://tools.genome-engineering.org), and sequences of the single guide RNA for CRISPR/Cas9 are: LXRα, 5′-TCGGCTTCGCA AATGCCGTC-3′; LXRβ, 5′-ACCCCGGCAGGCATAGCGCC-3′. The cells were switched to serum-free medium and received treatment when the confluence was ~90%.

In vivo study

The mouse in vivo study was approved by the ethics committee of Hefei University of Technology and was consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Animal studies are reported in compliance with the ARRIVE guidelines [30, 31]. C57BL/6 wild-type mice (~10-week, female) were randomly divided into two groups (five mice/group) and were injected intraperitoneally (ip) with corn oil (control group) or corn oil containing T317 (5 mg/kg) for 10 d. After treatment, the animals were anesthetized and euthanized in a carbon dioxide chamber. Then, liver samples were collected.

To determine whether T317 can inhibit HCC in vivo, we randomly divided BALB/c-nude mice (5 weeks, male) into two groups ($n = 6$). All mice were inoculated subcutaneously (sc) with Huh-7 cells (5×10^6 cells/mouse in 100 μL PBS) into the right axilla. When palpable tumors had formed, the animals were ip injected with corn oil (control group) or corn oil containing T317 (15 mg/kg) for 21 d, and tumor volume was measured every 3 d. At the end of the study, the animals were anesthetized and euthanized in a carbon dioxide chamber. Then, tumor samples were collected.

Western blot analysis

After treatment, total proteins were extracted from cells or a piece of liver followed by determination of REPS2, LXRα, LXRβ, EGFR, p-EGFR, AKT, p-AKT, ERK1/2, p-ERK1/2, NF-κB, p-NF-κB, p38MAPK, and p-p38MAPK protein expression by Western blots with quantitative analysis of band density based on three repeated experiments as described previously [32].

cDNA synthesis and quantitative real time-PCR (qRT-PCR)

After treatment, total cellular RNA was extracted from cells or mouse liver followed by determination of mRNA expression by quantitative real-time PCR (qRT–PCR) with a reverse transcription kit (New England Biolabs, Ipswich, MA), a SYBR Green PCR master mix (Vazyme Biotech, Nanjing, China) and the corresponding primers. The expression levels of REPS2, LXRα, and LXRβ mRNA were normalized to GAPDH or β-actin mRNA in the corresponding samples. The primers were as follows: homo-GAPDH: forward, 5′-ACAACTTTGGCATTGTGAA-3′, and reverse, 5′-GATGCAGGGATGATG TTCTG-3′; mus-β-actin: forward, 5′-ATGGAGGGGAATACAGCCC-3′, and reverse, 5′-TTCTTTGCAGCTCCTTCGTT-3′; homo-LXRα: forward, 5′-GAAGAAACTGAAGCGGCAAGA-3′, and reverse, 5′-ACTCGAAGCC GGTCAGAAAA-3′; homo-LXRβ, forward, 5′-TGCCTGGTTTCCTGC AGCT-3′, and reverse, 5′-AGATGTTGATGGCGATGAGCA-3′; homo-REPS2: forward, 5′-TGGACCAAAATAACCAACCC-3′, and reverse, 5′-GTGGCTGACCTGTTTCGG-3′.

Immunofluorescence staining

After treatment, the protein expression of REPS2 and LXRα in HepG2 and Huh-7 cells or the liver was determined by immunofluorescence staining [33]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min, followed by washing with PBS for 5 min. The cells or frozen liver sections were incubated with 0.1% Triton X100 for 10 min to break the membrane. After the samples were washed with PBS for 10 min, they were blocked with 2% BSA for 2 h. Then, the samples were incubated with primary antibody (1:200 dilution) for 18 h at 4 °C, followed by incubation with either rhodamine- or FITC-conjugated secondary antibody (1:1000 dilution) for 2 h. The nucleus of the samples were also stained with DAPI solution. Then cells or frozen sections were observed under a fluorescence microscope (Zeiss, Germany), and the images were photographed.

Immunohistochemistry (IHC)

A total of 10 HCC tissues and the corresponding adjacent noncancerous specimens were obtained from the Second Hospital of Anhui Medical University (Hefei, China). All samples were embedded in paraffin. Then, they were cut at 5 μm thickness. Tumor sections were immunostained with anti-REPS2 and LXRα antibodies by using Histostain-Plus Kits from Beijing Zhongshan Biotechnologies (SP-90001) according to the manufacturer's instructions.

Construction of normal or mutated REPS2 promoter(s), and determination of promoter activity

The human REPS2 promoter (from −1687 to −921) sequence was generated by PCR using HepG2 cell genomic DNA as a template and the following primers: forward, 5′-ATATGTGGGCCTGATGCTG GAAC-3′, reverse, 5′- CAAATGTGCTTGCTTCCCCCTGG-3′. The PCR product was inserted into the luciferin reporter vector of pGL4.10, and the constructed promoter was named pREPS2. Site-directed mutagenesis of the predicted LXRE sequence was performed as shown in Fig. 3c. Primers were designed and the mutant promoter was constructed and named pREPS2 mut.

To determine the effect of LXR activation on REPS2 promoter activity, we transfected pREPS2 or pREPS2 mut and the internal reference plasmid (pGL4.70) into Huh7 cells with Lipofectamine transfection reagent. After transfection for 6–8 h, the cells were treated with T317 for 18 h. Firefly and Renilla luciferase activities were measured in cell lysates using the dual luciferase reporter assay system.

Chromatin immunoprecipitation (ChIP) analysis

To determine the binding activity of the LXR protein with LXRE in the REPS2 promoter, we treated HepG2 cells with T317 (400 nM) for 18 h. The protein/DNA complex was crosslinked with 1% formaldehyde for 15 min, and then glycine (0.125 M) was added to stop the crosslinking. The nuclear protein was extracted by a Nuclear Protein Extraction Kit (KeyGen Biotech, Nanjing, China). The chromatin was then broken into 200–1000 bp fragments by ultrasound. The immunoprecipitation was conducted with the same amount of chromatin from each sample based on the input and the specific LXRα or LXRβ monoclonal antibody separately. The relative amount of gene fragments binding with LXR was tested by qPCR. The primers for the CHIP assay were as follows: forward, 5′-TCAAGCCTGTAATCCCAGCACTTT-3′, reverse, 5′-GGCTG TAGTTCAATGGCACAGTCTT-3′.

Wound healing assay

HepG2 or Huh7 cells were transfected with REPS2 siRNA (siREPS2) or control siRNA for 48 h. The same amount of transfected cells was seeded into 6-well plates at a density of 1×10^6/mL. After adhesion, the cells were treated with or without T317 (0.5 μM) for 18 h. A wound gap was scratched using a 200 μL pipette tip followed by three gentle washes with PBS to remove cellular debris. The cells were incubated in serum-free medium for 48 h and recorded every 24 h. Images were acquired using a microscope with a 5× objective. The wound healing rates were calculated using ImageJ software.

Colony formation assay

HepG2 or Huh7 cells were transfected with REPS2 siRNA (siREPS2) or control siRNA for 48 h. The transfected cells were seeded in 6-well plates at a density of 2000 cells per well. The cells were stable for 3–4 h, treated with T317 (0.5 μM) and cultured continuously for several days at 37 °C until colonies were visible. The colonies were then stained with a 0.5% crystal violet solution for 5~10 min. The cells were washed with PBS to clear the remaining dye solution. A digital camera was used to take pictures. Crystal violet was extracted from the cells by adding 33% acetic acid aqueous solution to each well, and 100 μL was aspirated and transferred to a 96-well plate. Finally, the absorption peak was detected at 570 nm.

Immunofluorescence and quantitative analysis of receptor endocytosis

After immunofluorescence staining, the process of EGF-mediated EGFR endocytosis was observed by laser confocal microscopy, and receptor endocytosis was analyzed quantitatively [34]. Briefly, two polygons were drawn along the outer edge of the cell membrane and the outer edge of the nucleus to obtain the fluorescence intensity and area of the whole cell, and then the mean fluorescence degree of the cell membrane ($mean_{mem}$) was calculated. Statistical analyses were performed using ImageJ software, and the mean fluorescence intensity of cells without EGF stimulation was considered the total cellular fluorescence intensity ($mean_{total}$). Therefore, the percentage of internalized receptors by cells was calculated as follows: internalized receptor (percentage) = $(1\text{-}mean_{mem}/mean_{total}) \times 100\%$. For each value, at least 6 cells from different fields were analyzed.

Data analysis

All experiments were repeated at least three times, and representative results are presented. Data are presented as the mean ± standard error. All the data were initially analyzed for normality and equal variance as a justification for using parametric or nonparametric analyses using SPSS software. Statistical significance was evaluated using an unpaired two-tailed Student's *t* test for two groups or one-way ANOVA with a post hoc test among more than two groups using Prism 5 (GraphPad Software, Inc.). A significant difference was defined as $P < 0.05$.

RESULTS

Activation of LXR induces REPS2 protein expression in hepatic cell lines

Both LXR and REPS2 are key regulators of the development and progression of different tumors [35, 36]. LXR inhibits the growth of HCC cells, which also serves as a prognostic marker for patients with HCC. However, the role and regulatory relationship between LXR and REPS2 in HCC is unknown. To evaluate the effect of LXR activation on REPS2 expression in HCC cells, we initially treated HepG2 cells with different concentrations of a synthetic LXR ligand, T317, for 18 h. The results showed that T317 induced REPS2 protein expression in HepG2 cells in a concentration-dependent manner, with the maximum induction at 0.4 μM (Fig. 1a, left upper panel). To further validate whether the regulation of REPS2 by LXR was cell- or species-dependent, we treated BNLCL.2 (a murine normal liver cell line), Huh-7 (another human HCC cell line) and LO2 (human fetal hepatocyte line) cells with T317. As shown in Fig. 1a, T317 also significantly induced REPS2 protein expression in BNLCL.2, Huh-7 and LO2 cells, indicating that LXR induced REPS2 expression in normal hepatocytes and HCC cells both in humans and rodents.

To further confirm that LXR activation induced REPS2 protein expression in hepatocytes, we performed immunofluorescence staining of intact HepG2 and Huh-7 cells after T317 treatment. Similarly, T317 markedly increased REPS2 protein expression in HepG2 and Huh-7 cells (Fig. 1b).

LXR expression is essential for the induction of REPS2 expression

To determine whether the induction of REPS2 expression by T317 is directly mediated by LXR, we transfected HepG2 cells with an LXRα or LXRβ overexpression vector. Compared with the control, overexpression of LXRα or LXRβ protein increased REPS2 protein expression in HepG2 cells (Fig. 2a). Conversely, we knocked down the expression of LXRα or LXRβ by specific siRNA in HepG2 or Huh-7 cells, respectively. As expected, along with reduced LXRα or LXRβ expression, the expression of REPS2 was reduced (Fig. 2b, c). Thus, REPS2 can be induced in an LXR-dependent manner.

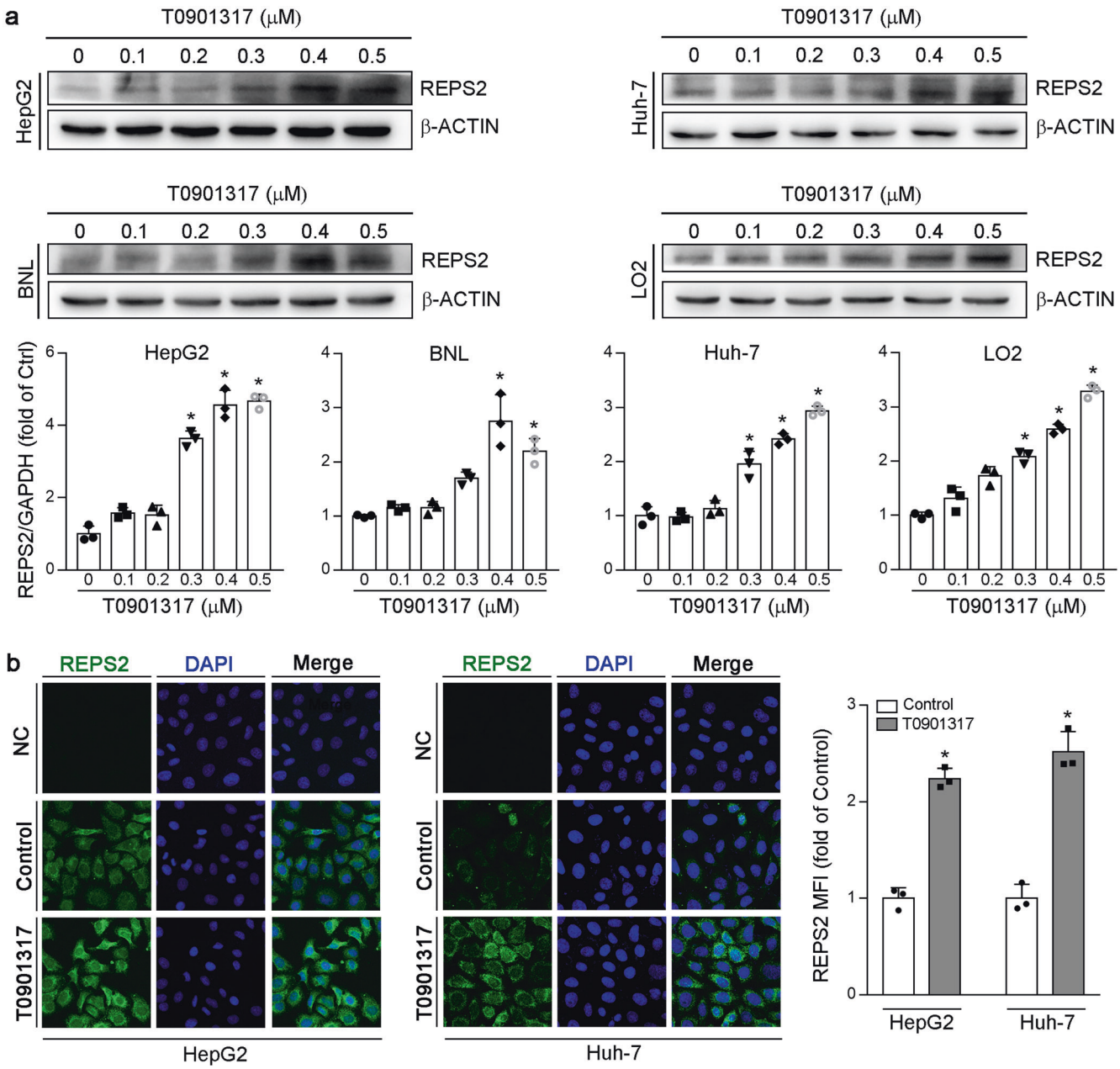


Fig. 1 LXR activation induces REPS2 protein expression in hepatocytes. a HepG2, BNLCL.2 (BNL), Huh-7 and LO2 cells were treated with T317 at the indicated concentrations for 18 h. The expression of REPS2 was determined by Western blotting. The band was quantitively analyzed by ImageJ. *$P < 0.05$ versus the controls ($n = 3$). **b** HepG2 or Huh-7 cells at ~60% confluence were treated with T317 (400 nM) in serum-free medium for 18 h. REPS2 protein expression in both cell lines was assessed by immunofluorescence staining. *$P < 0.05$ versus the controls ($n = 3$).

T317 induces REPS2 expression in an LXRE-dependent manner

To determine whether LXR-induced hepatocyte REPS2 expression is accomplished transcriptionally, we examined the changes in REPS2 mRNA expression in hepatocytes after T317 treatment. The results in Fig. 3a show that REPS2 transcripts were significantly increased after T317 treatment in HepG2, BNLCL.2, Huh-7 and LO2 cells, indicating that the regulation may be at the transcriptional level.

Then, we constructed a REPS2 promoter (pREPS2, from −1687 to −921). T317 induced REPS2 promoter activity in a concentration-dependent manner in LO2 cells (Fig. 3b). By sequence alignment analysis, we found a putative LXRE in the REPS2 promoter region with the sequence of GGATCAcctgAGGTCA (Fig. 3c, from −1209 to −1193), which was very similar to the conserved sequence of LXRE (AGGTCAn$_4$AGGTCA). Therefore, we speculated that REPS2 is a direct target gene of LXR. To further determine the importance of this putative LXRE in REPS2 transcription, we constructed a REPS2 promoter with the putative LXRE mutation on the basis of pREPS2 (pREPS2 LXRE mut or pREPS2 mut, Fig. 3c). LXRE mutation reduced the basal REPS2 promoter activity, and T317 failed to increase the pREPS2 mut promoter activity (Fig. 3d), indicating that LXR-induced REPS2 transcription depended on the presence of LXRE in the REPS2 promoter. Additionally, chromatin immunoprecipitation assays demonstrated that T317 significantly increased the binding of LXRα and LXRβ proteins to LXRE in the REPS2 promoter. Thus, the results above suggest that LXR induces REPS2 expression at the transcriptional level by binding to LXRE in the REPS2 promoter region.

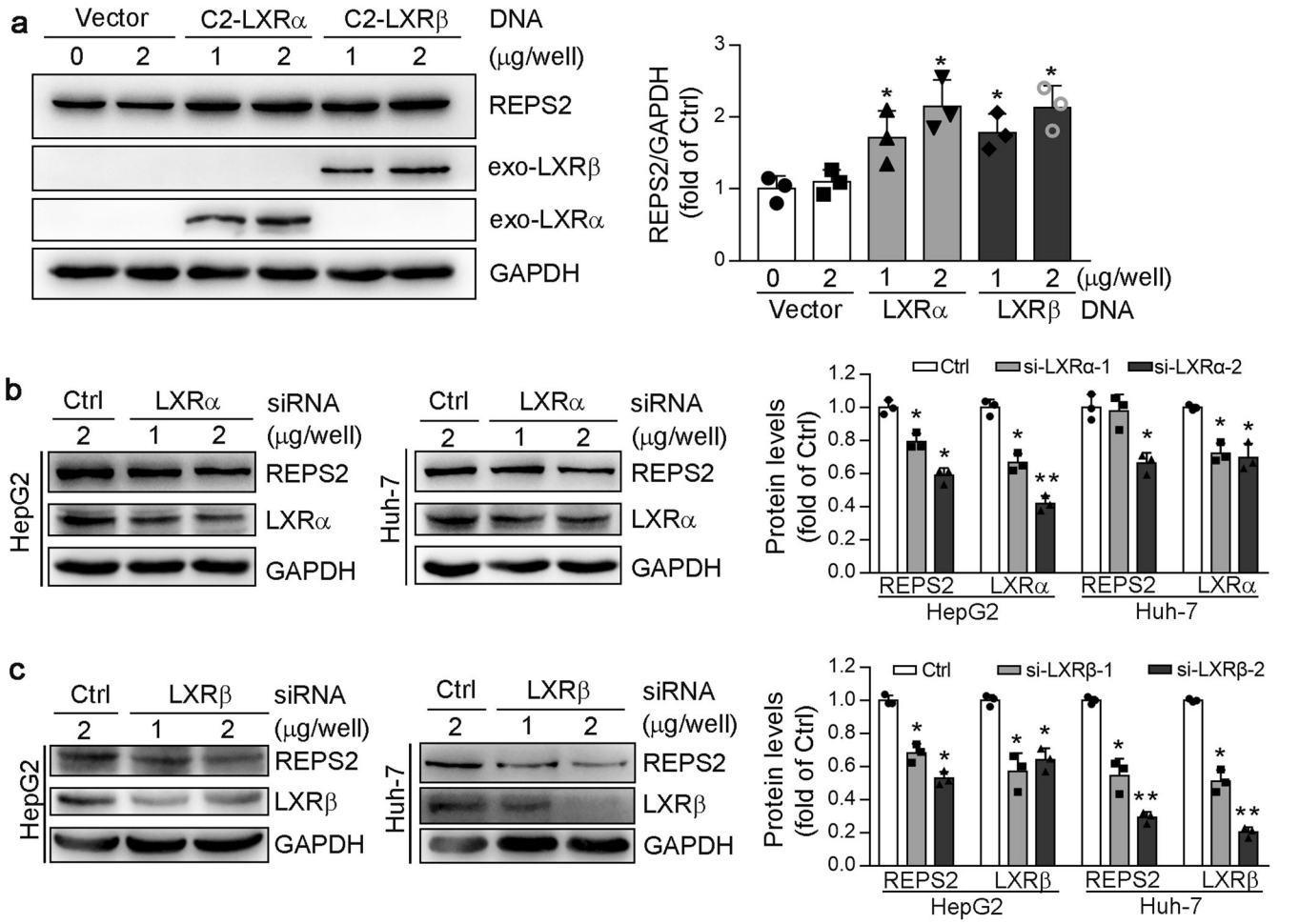


Fig. 2 LXR expression is essential for T317-induced REPS2 expression. a HepG2 cells in 6-well plates were transfected with plasmid DNA of pEGFP-C2, pEGFP-LXRα or pEGFP-LXRβ expression vectors at the indicated concentrations for 24 h. **b**, **c** HepG2 or Huh-7 cells were transfected with si-LXRα or si-LXRβ for 48 h at the indicated concentrations. At the end of treatment, total cellular protein was extracted and used to determine LXRα, LXRβ and REPS2 expression by Western blots. The band was quantitively analyzed by ImageJ. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the controls ($n = 3$).

Activation of LXR induces the production of REPS2 in vivo

To determine the physiological relevance of LXR activation on REPS2 expression, we ip injected C57BL/6J mice (10~week old female; $n = 5$) with corn oil (control group) or corn oil containing T317 (5 mg/kg) for 10 d. Along with increased LXRα and LXRβ protein and mRNA expression, T317 significantly increased REPS2 protein and mRNA levels in the liver (Fig. 4a, b). Immunofluorescence staining confirmed that T317 induced LXRα expression and nuclear translocation (left panel, Fig. 4c). Consistently, hepatic REPS2 expression was also enhanced by T317 treatment (right panel, Fig. 4c). In summary, the data in Fig. 4 demonstrate that LXR regulates REPS2 expression in mouse livers.

T317 inhibits proliferation and migration of HCC via induction of REPS2 expression

To investigate the relationship between REPS2 and HCC, we first analyzed the expression of REPS2 in HCC tissues using the TNMplot database (TNMplot.com) [37]. The expression of REPS2 in HCC tumor tissues was significantly lower than that in normal liver tissues (Fig. 5a). Then, we analyzed the prognosis of HCC with REPS2 expression by the Kaplan–Meier plotter database. The 10-year survival rate of patients with low REPS2 expression was lower than that of patients with high REPS2 expression (Fig. 5b). Immunohistochemistry showed that REPS2 and LXRα proteins expression was significantly downregulated in HCC tumor tissues compared with adjacent normal liver tissues (Fig. 5c). We also observed that the mRNA levels of REPS2 in HCC cell lines (HepG2) were lower than those in the normal liver cell line (LO2) and were further reduced by LXRα or LXRβ deficiency (Fig. 5d). These results suggest that REPS2 is inversely correlated with the development of HCC and that reduced REPS2 expression is associated with poor prognosis, indicating that REPS2 may be involved in the development of HCC.

To explore the role and function of REPS2 in HCC, we detected the migration and colony formation of HCC cells by T317 treatment with or without REPS2 siRNA transfection. As shown in Fig. 5e, T317 significantly inhibited cell migration in HepG2 and Huh-7 cells, and REPS2 knockdown restored the T317-induced anti-migratory effects. Moreover, the T317-inhibited clonogenic capacity was recovered by REPS2 siRNA in HepG2 and Huh7 cells (Fig. 5f). These results suggest that LXR inhibits HCC cell migration and clonogenicity by upregulating, at least partially, REPS2 expression.

To determine the antitumor effect of T317 in vivo, we injected Huh-7 cells into nude mice with or without T317 administration. Compared with the controls, the mice treated with T317 had no significant weight loss or other observable side effects. However, the development of xenograft tumors of Huh-7 cells was

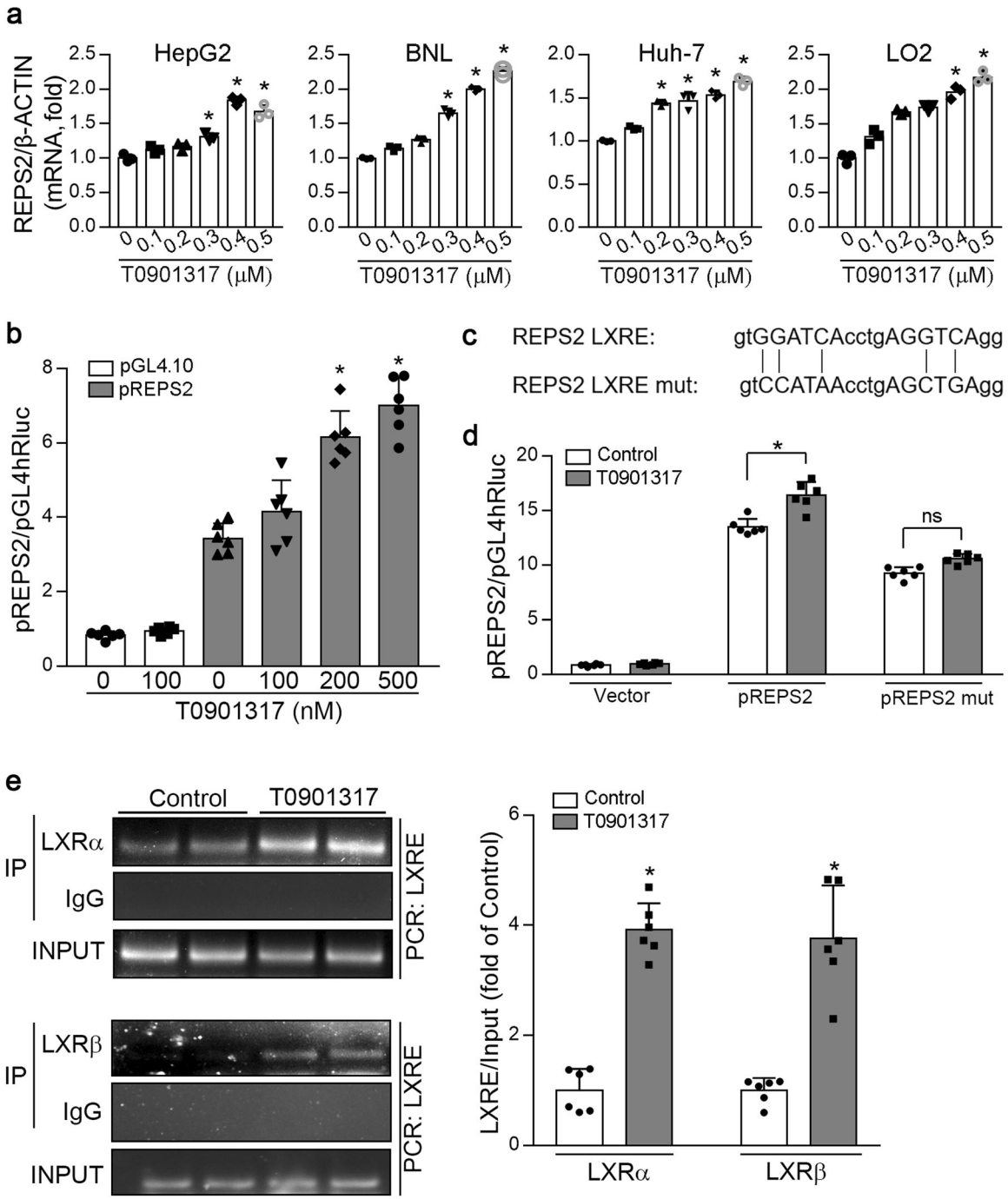


Fig. 3 REPS2 expression is induced in an LXRE-dependent manner. a HepG2, BNL, Huh-7 and LO2 cells were treated with T317 at the indicated concentrations for 18 h. Total cellular RNA was extracted and used to determine REPS2 mRNA expression by qRT–PCR. *$P < 0.05$ ($n = 3$); **b** LO2 cells were transfected with the REPS2 promoter cloned into the pGL4.10 reporter (pREPS2, 766 bp) for 12 h. Then, the cells were treated with T317 at the indicated concentrations for 18 h. The cell lysates were collected, and the luciferase activity was measured using a dual luciferase reporter assay kit. *$P < 0.05$ ($n = 3$); **c** the predicted LXRE sequence in upstream of REPS2 (upper panel) and the corresponding mutation sequence of the REPS2 promoter (lower panel, pREPS2 mut); **d** pGL4.10 vector, pREPS2, or pREPS2 mut was transfected into HepG2 cells for 12 h. Then, T317 (400 nM) was added, and the cells were treated for 18 h. Luciferase activity was detected. *$P < 0.05$ versus the controls ($n = 6$); **e** HepG2 cells were treated with T317 (400 nM) for 12 h, and the chromatin DNA of HepG2 cells was extracted and subjected to CHIP with the addition of IgG (negative control), LXRα antibody or LXRβ antibody, and the corresponding primers were used to amplify the fragment of interest. *$P < 0.05$ versus the controls ($n = 6$).

significantly inhibited by T317 (Fig. 5g, h). Consistent with the findings in C57BL/6 mice, T317 also increased the mRNA levels of LXRα/β and REPS2 in the tumor tissues (Fig. 5i). Taken together, the results in Fig. 5 suggest that the LXR agonist T317 markedly inhibited the growth of HCC both in vitro and in vivo.

Upregulation of REPS2 by LXR inhibits EGF-induced endocytosis of EGFR

REPS2 was suggested to be involved in EGF-induced EGFR endocytosis, which is an essential step for EGF-induced cell proliferation and migration. To determine whether LXR reduced

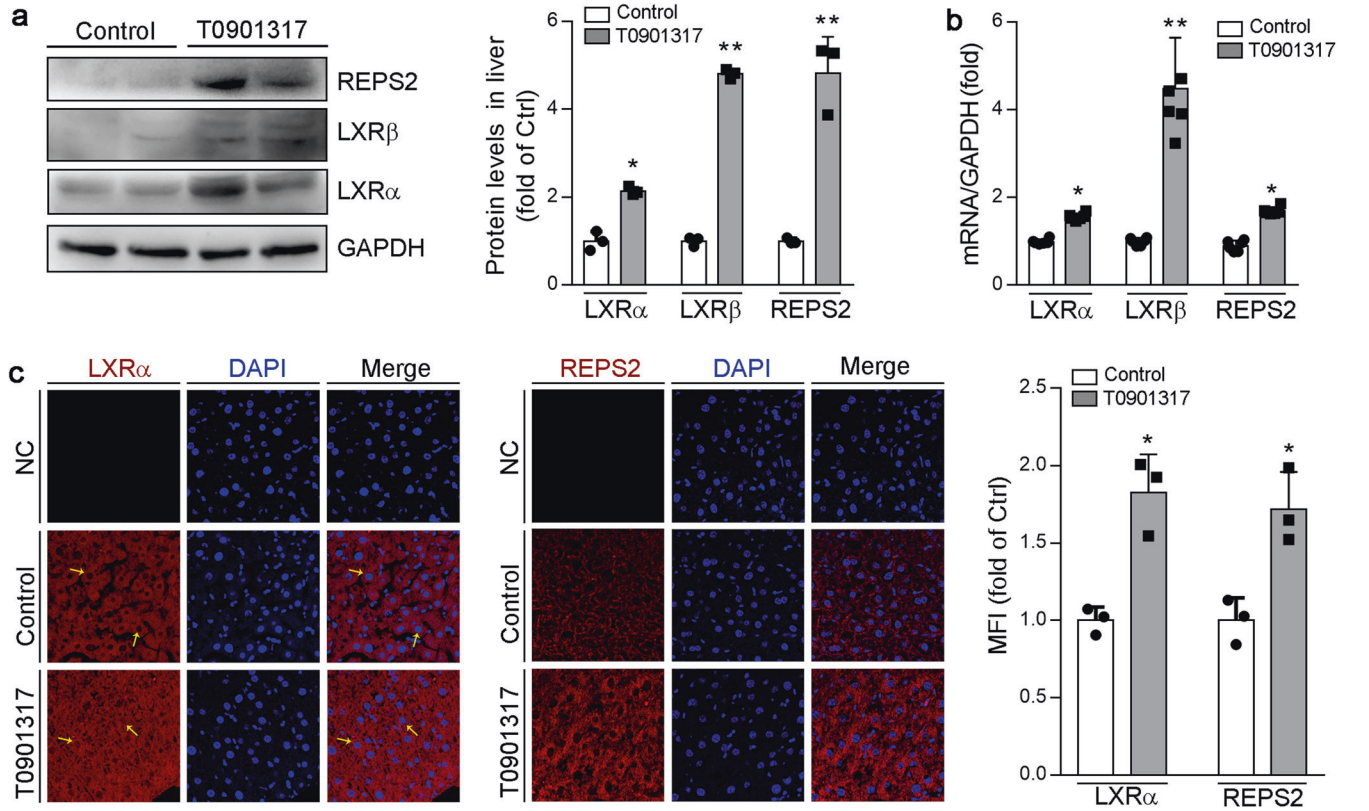


Fig. 4 Activation of LXR induced the production of REPS2 in vivo. C57BL/6 J wild-type mice (~10 weeks old, females, $n = 5$) were injected ip with corn oil (control group) or corn oil containing T317 (5 mg/kg) for 10 d. After treatment, the following analyses were completed: The protein (**a**) and mRNA (**b**) expression of REPS2, LXRα and LXRβ in the liver were detected by Western blots and qRT–PCR, respectively. REPS2 and LXRα protein expression in the liver was also determined by immunofluorescence staining (**c**). $^{*}P < 0.05$; $^{**}P < 0.01$ versus the controls ($n = 3$).

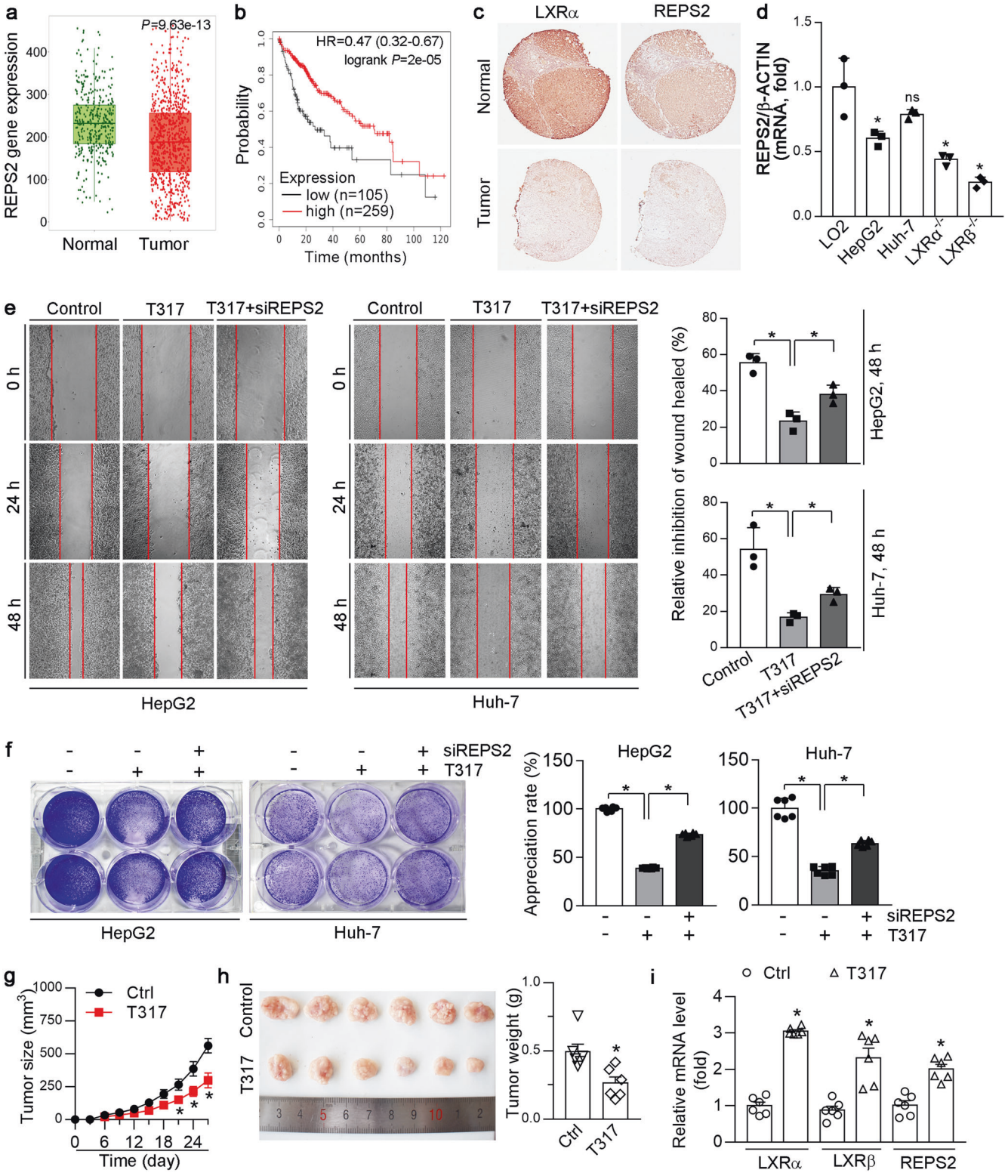
HCC cell migration and clonogenicity via REPS2-mediated EGFR endocytosis, we assessed the endocytosis of EGFR in the presence of EGF. EGF rapidly stimulated the endocytosis of EGFR, and more than 50% of membrane EGFR was endocytosed after EGF treatment at 30 min (upper panel, Fig. 6a). T317 treatment reduced the endocytosis of EGFR, which resulted in ~70% EGFR retention in the membrane after T317 treatment at 30 min (middle panel, Fig. 6a). However, the T317-inhibited endocytosis was recovered by REPS2 knockdown (bottom panel, Fig. 6a). Additionally, we determined that LXRα knockout in HepG2 cells accelerated the EGF-induced EGFR endocytosis rate (Fig. 6b). Taken together, the data in Fig. 6 suggest that LXR activation reduced EGFR endocytosis by upregulating REPS2 expression.

Induction of REPS2 expression by T317 reduces the maintenance of EGFR-mediated signaling

EGFR is a transmembrane tyrosine kinase receptor that can be activated by a variety of ligands, which in turn activate multiple signaling pathways that promote tumor cell proliferation and metastasis and inhibit apoptosis. REPS2 was identified as a binding protein of RalBP1 and is thought to be involved in the EGFR signaling pathway. In HepG2 cells, EGF treatment induced the phosphorylation of EGFR (p-EGFR) at peak times at approximately 0.5 h and then the level of p-EGFR declined gradually (Fig. 7a). However, T317 increased the p-EGFR level at 0.5 h but decreased it thereafter. Knockdown of REPS2 expression had no effect on the T317-increased p-EGFR level at 0.5 h, while it substantially inhibited the T317-reduced p-EGFR levels after 1 h (Fig. 7a).

Many studies have shown that the EGFR-PI3K-AKT signaling axis plays an important role in the malignant progression of HCC. The EGFR-PI3K-AKT pathway can further activate NF-κB through various mechanisms. Compared with the control, T317 induced higher phosphorylation of AKT (p-AKT) at 30 min, followed by a rapid decrease, which was barely detected after 2 h (Fig. 7b), indicating the inhibition of the AKT signaling pathway by T317 for long-term treatment. Accordingly, the phosphorylation of NF-κB (p-NF-κB) was inhibited by T317 at 2 h. However, the knockdown of REPS2 by siRNA abolished the effect of T317 on the expression pattern of p-AKT and p-NF-κB. Moreover, LY294002, a PI3K inhibitor, abolished effects of siREPS2 on T317-mediated reductions in p-AKT and p-NF-κB activation (Fig. 7c), indicating that T317 inhibited the EGFR-PI3K-AKT-NF-κB signaling pathway by upregulating REPS2 expression.

The p38MAPK and ERK1/2 signaling pathways, which are activated by signal transduction from cell surface receptors such as receptor tyrosine kinases (RTKs) or G protein coupled receptors (GPCRs), are frequently activated in liver cancer. Improper regulation of pathways leads to aberrant cell behavior, including increased cell growth and proliferation, dedifferentiation, and survival, which all contribute to carcinogenesis. Figure 7d indicates that EGF stimulated p-ERK1/2 at 30 min, and the increase was observed at 2 h. However, p-ERK1/2 was rapidly decreased by T317 after 1 h and disappeared at 2 h. In contrast, the p-ERK1/2 was gradually increased in the presence of REPS2 siRNA (Fig. 7d). Similar results were also observed for the expression of p-p38MAPK. The effect of REPS2 siRNA on T317-inhibited p-p38MAPK and p-ERK1/2 was reversed by SB203580 (p38MAPK inhibitor) and U0126 (MEK1/2 inhibitor) (Fig. 7e). These results indicated that T317 could also upregulate REPS2 expression to inhibit the EGFR-MAPK signaling pathways.



DISCUSSION

HCC is a malignant tumor with high morbidity and mortality rates [38]. Nuclear receptors are important regulators at the transcriptional level and are involved in the regulation of multiple biological processes. Increasing evidence has shown the functional role of LXRs in a variety of malignant tumors and the potential therapeutic effects of their ligands [36]. The LXR agonist T317 reduced the intratumoral abundance of regulatory T cells (Tregs) and the expression of the Treg-attracting chemokine Ccl17 by MHCII^{high} tumor-associated macrophages (TAMs) [39]. Withaferin A, a potential ligand for LXRα, was reported to inhibit HCC by reducing NF-κB target gene expression [40, 41]. In this study, we confirmed that LXR agonists could inhibit the proliferation and migration of the

Fig. 5 T317 inhibited the proliferation and migration of HCC cells by inducing REPS2 expression. a The TNMplot database was used to analyze REPS2 levels in HCC and normal liver; **b** The Human Protein Atlas database was used to analyze the relationship between REPS2 expression and prognosis; **c** Immunohistochemistry analysis of REPS2 and LXRα protein in paraffin-embedded clinical HCC tissues and the corresponding adjacent normal tissues; **d** qRT–PCR analysis of REPS2 mRNA levels in LO2, HepG2, Huh-7, HepG2LXRα$^{-/-}$ and HepG2LXRβ$^{-/-}$ cells; *$P < 0.05$ versus the LO2 group ($n = 3$); **e** HepG2 or Huh-7 cells in 12-well plates were transfected with shRNA against REPS2 or control shRNA (2 μg/well) for 48 h. Then, the cells were treated with or without T317 (0.5 μM) for 18 h. Wound healing assays were used to determine the migration of HepG2 or Huh-7 cells. *$P < 0.05$ ($n = 3$); **f** HepG2 or Huh-7 cells in 6-well plates (2000 per well) were transfected with shRNA against REPS2 or control shRNA (2 μg/well) for 48 h. Then, the cells were treated with or without T317 (0.5 μM). Colony formation assays and crystal violet staining were used to detect the cell colony formation of HepG2 or Huh-7 cells. *$P < 0.05$ ($n = 3$); **g–i** 5×10^6 Huh-7 cells were implanted into the right axilla of each 5 week-old male nude mouse. When palpable tumors were formed, T317 (15 mg·kg^{-1}·d^{-1}) or vehicle (corn oil) was ip injected every day for 21 d ($n = 6$). The tumor volume was measured every 3 d (**g**). Then, the mice were sacrificed and the xenograft tumors were weighed and photographed (**h**). The mRNA expression of REPS2, LXRα and LXRβ in the tumor was determined by qPCR (**i**). *$P < 0.05$ ($n = 6$).

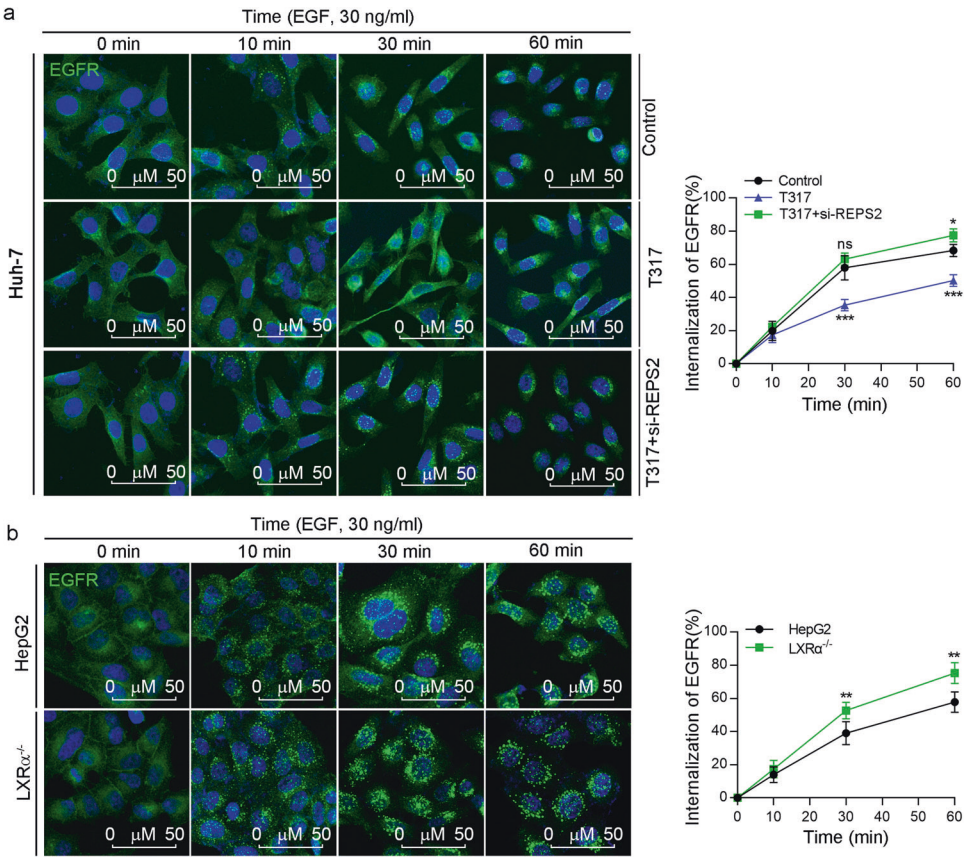


Fig. 6 Induction of REPS2 expression by T317 inhibits EGF mediated endocytosis of EGFR. a HepG2 cells were transfected with siRNA against REPS2 for 48 h, followed by treatment with or without T317 (400 nM) for 18 h. Then, HepG2 cells were serum-starved and treated with 30 ng/mL EGF for the indicated amounts of time. **b** LXRα knockout cells (LXRα$^{-/-}$) and control HepG2 cells were serum-starved and treated with 30 ng/mL EGF for the indicated time. The endocytosis of EGFR (green) was determined by immunofluorescence. Data were quantified as described in "Materials and methods". *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus the controls or HepG2 cells ($n = 6$, Student's t test).

HCC cell lines HepG2 and Huh-7. Moreover, T317 could inhibit the development of Huh-7 xenografts in nude mice.

REPS2 is an important player in receptor-mediated endocytosis including the Ras/Ral signaling pathway [16]. REPS2 contains an Eps15 homology (EH) domain and proline-rich structural domains that are important for molecular signaling and receptor endocytosis through protein-protein interactions [18, 42]. REPS2 also inhibits RalBP1-mediated transport of chemotherapeutic drugs (adriamycin) out of cells to increase drug sensitivity [43]. REPS2 is also dysregulated in the development of prostate cancer and is a potential biomarker for breast and prostate cancers [19, 21]. Given the important role of REPS2 in signal transduction, it is crucial to explore its upstream regulatory mechanisms. In the present study, we found that REPS2 was stably downregulated in LXRα or LXRβ knockdown HepG2 cells and upregulated by LXR agonists. Then, by dual-luciferase reporter assays and ChIP assays, we found that LXR regulates REPS2 expression by directly binding to its promoter sequences.

Given the role of REPS2 in multiple cancers but not HCC, we explored the changes in REPS2 expression in normal paired tissues and liver cancer tissues using the TNMplot database. The results suggest that the total expression of REPS2 in tumor tissues was decreased and might be associated with the development of HCC. In human HCC patients, LXRα and REPS2 were reduced in the tumor areas. In vitro, REPS2 expression was higher in normal human-derived hepatocyte LO2 cells than in liver cancer cells. Survival analysis using the human protein atlas database indicated that low levels of REPS2 were associated with poor prognostic survival. Interestingly, the LXR agonist T317-inhibited HCC cell development was diminished when REPS2 expression was

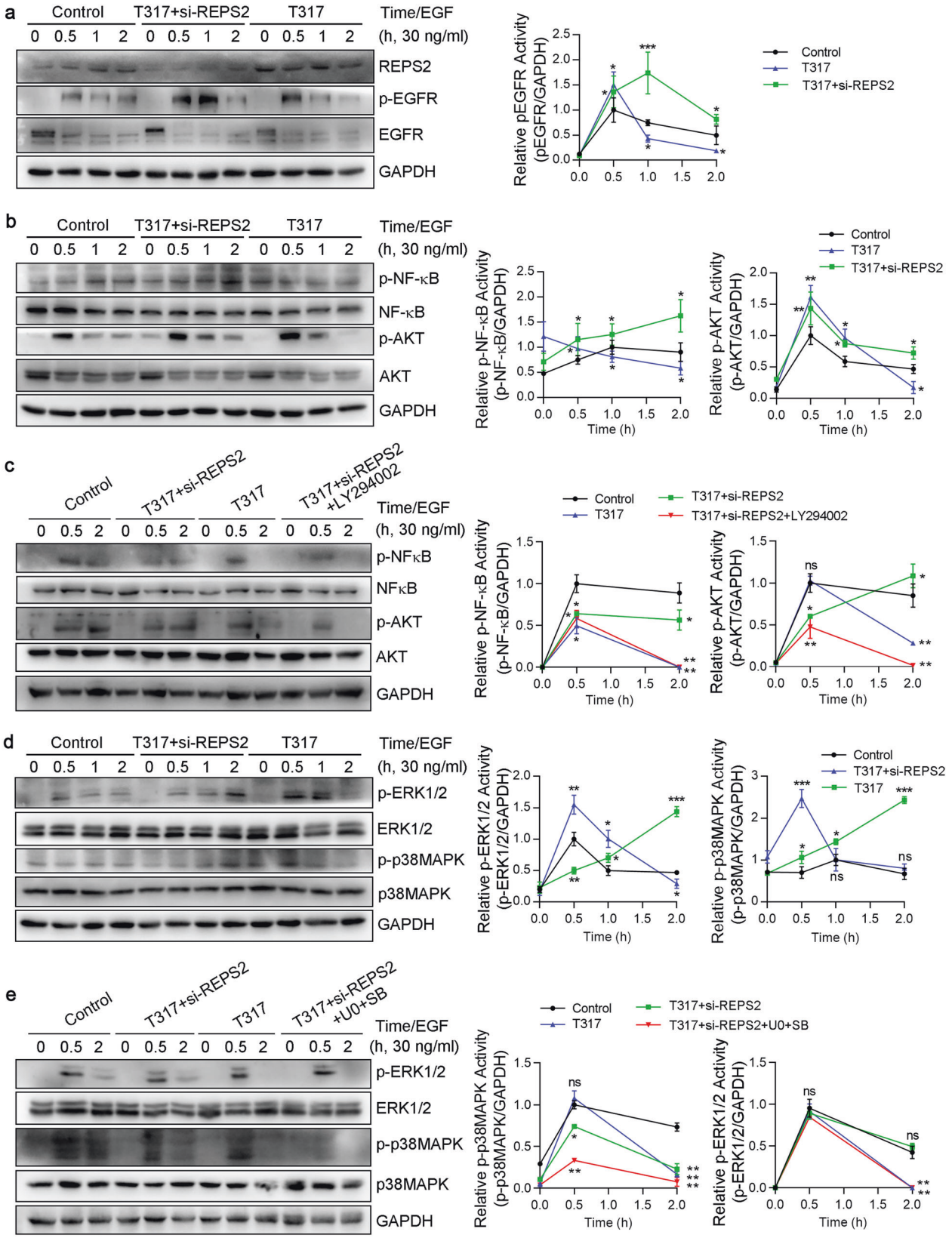


Fig. 7 Induction of REPS2 expression by T317 reduces the maintenance of EGFR-mediated signaling. a–e HepG2 cells were transfected with siRNA against REPS2 for 48 h, followed by treatment with or without T317 (400 nM), LY294002 (10 μM) or U0126 (U0, 2 μM) plus SB203580 (SB, 5 μM) for 18 h. Then, HepG2 cells were serum-starved and treated with 30 ng/mL EGF for the indicated time. At the end of the treatment, total cellular protein was extracted. Western blotting was used to detect REPS2, p-EGFR, EGFR (**a**), p-NF-κB, NF-κB, p-AKT, AKT (**b**, **c**), p-ERK1/2, ERK1/2, p-p38MAPK and p38MAPK (**d**, **e**) protein expression. The band was statistically analyzed by ImageJ software, and the expression of GAPDH was calculated as an internal control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the controls ($n = 3$).

decreased, indicating that REPS2 is closely related to T317-inhibited tumor proliferation and migration.

EGFR is a transmembrane protein receptor with tyrosine kinase activity [44]. After binding with ligands (such as EGF), EGFR automatically phosphorylates at specific sites to form a homodimer to contact various signaling pathway proteins [45]. After activation, EGFR is endocytosed into the cells for recycling. We determined that EGF-induced EGFR endocytosis was reduced by LXR activation but increased by LXRα knockout. However, the reduced EGFR endocytosis was restored by REPS2 knockdown, indicating that REPS2 is essential for LXR-regulated EGFR endocytosis. Interestingly, previous studies have shown that LXRβ may sensitize HCC to sorafenib by inhibiting EGFR [46]. Both LXRα and LXRβ can induce REPS2 expression. The expression of REPS2 was lower in LXRβ$^{-/-}$ HepG2 cells, indicating that LXRβ may be the main contributor to the induction of REPS2 expression. As shown by LXRα-induced fatty liver disease [47], targeting LXRβ may be an interesting strategy to limit HCC in the future.

The PI3K/AKT/NF-κB, p38MAPK and ERK1/2 signaling pathways are involved in regulating the cell cycle, cell proliferation, apoptosis, metabolism and angiogenesis and are dysregulated in many cancer types including HCC [48, 49]. In this study, the phosphorylation level and time of EGFR decreased significantly after T317 treatment. The corresponding result is also reflected in the phosphorylation level and time of key proteins in the PI3K/AKT/NF-κB, p38MAPK and ERK1/2 signaling pathways. The results indicated that T317 exerted its antitumor effect by upregulating REPS2 expression and inhibiting EGFR endocytosis and its downstream signaling pathways. Interestingly, we determined that T317 induced acute EGFR phosphorylation at 0.5 h and thereafter inhibited it. EGFR dynamics affect its function. Compared with EGF, epiregulin and epigen induce weaker and more short-lived EGFR dimers which causes sustained EGFR signaling to induce MCF-7 cell differentiation but not proliferation [50]. A previous study also showed that LXR agonist has a limited effect on MHCC97H cell (a human HCC cell line) proliferation but sensitizes it to sorafenib [46]. We determined that the LXR agonist inhibited HCC cell (HepG2 and Huh-7) migration and colony formation. These results may be related to the transient activation of EGFR by T317, which needs further study.

For the clinical therapeutics of HCC, except sorafenib, which has partial clinical benefit in advanced HCC, the overall clinical benefit of drugs targeting EGFR is not satisfactory. Drug resistance is an urgent problem in current HCC therapies due to the multiplicity of HCC disease mechanisms, as well as the high complexity and interactivity of the EGFR signaling pathway. In this study, the LXR pathway and EGFR pathway were linked through REPS2, which suggested new ideas for combining drugs to improve the effect of targeted therapy. However, it will be of interest to study how the reduced EGFR endocytosis inhibited its downstream signal transduction, and whether the LXR-inhibited HCC would be interrupted by REPS2 deficiency in vivo.

In conclusion, this study identified REPS2 as a direct undefined LXR target gene that is necessary for LXR-reduced HCC proliferation and migration. The anti-HCC effect of the LXR agonist involved, at least partially, REPS2-inhibited EGFR endocytosis and its downstream inhibition of the PI3K/AKT/NF-κB, p38MAPK, and ERK1/2 signaling pathways. Our study provides new insights into the potential mechanism and therapeutics of LXR agonists for HCC.

DATA AVAILABILITY

All data shown are available in the article and the supporting information.

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AUTHOR CONTRIBUTIONS

Conceptualization: YLC and JHH; methodology and investigation: XYH, MMZ, JZ, CYW, XKZ, SZ, XXY, and YJD; software: XYH and BTZ; clinical sample collection: DCZ; writing—original draft preparation: XYH and MMZ; writing—review and editing: YLC; supervision: YLC; funding acquisition: YLC, XXY, and JHH. All authors have read and agreed to the final version of the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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