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Curcumin Recovers Intracellular Lipid Droplet Formation Through Increasing Perilipin 5 Gene Expression in Activated Hepatic Stellate Cells *In Vitro*[*]

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Summary: The activation of hepatic stellate cells (HSCs) is a major event during hepatic fibrogenesis. Restoration of intracellular lipid droplet (LD) formation turns the activated HSC back to a quiescent state. Our previous studies have shown that curcumin suppresses HSC activation through increasing peroxisome proliferator-activated receptor, gamma (PPARγ) and 5' adenosine monophosphate-activated protein kinase (AMPK) activities. This study aims at evaluating the effect of curcumin on lipid accumulation in HSCs and hepatocytes, and further elucidating the underlying mechanisms. Now we showed that curcumin increased LD formation in activated HSCs and stimulated the expression of sterol regulatory element-binding protein and fatty acid synthase, and reduced the expression of adipose triglyceride lipase. Exogenous perilin5 expression in primary HSCs promoted LD formation. Perilipin 5 siRNA eliminated curcumin-induced LD formation in HSCs. These results suggest that curcumin recovers LD formation and lipid accumulation in activated HSCs by increasing perilipin 5 gene expression. Furthermore, inhibition of AMPK or PPARγ activity blocked curcumin's effect on Plin5 gene expression and LD formation. Our results provide a novel evidence *in vitro* for curcumin as a safe, effective candidate to treat liver fibrosis.
Key words: hepatic stellate cells; lipid droplets; perilipins; curcumin; liver fibrosis

Hepatic fibrosis refers to deposition of high-density extracellular matrix (ECM) protein and development of hepatic scar tissue caused by an imbalance between fibrogenesis and fibrolysis, which is the initial step in the development of chronic liver disease for cirrhosis and hepatocellular carcinoma[1, 2]. The major etiologies include viral hepatitis, alcoholic, and non-alcoholic steatohepatitis[3–5]. Till now, very few therapeutic strategies are effective in the intervention of this disease[6]. Since hepatic fibrosis is a dynamic and potentially reversible process[7, 8], the development of new rational and safe therapies targeting liver fibrogenesis still draws more attentions in hepatology.

Hepatic stellate cells (HSCs) are the main effectors in the development of liver fibrosis, regardless of etiology[9]. HSCs normally reside in the space of Disse in a quiescent, non-proliferative state. They are characterized by abundant lipid droplets (LDs), composed of retinyl esters, triglycerides, cholesteryl esters, cholesterol, phospholipids, and free fatty acids[10]. During hepatic injury, quiescent HSCs undergo the profound phenotypic changes, which is characterized by the loss of LDs. Recent studies demonstrated the importance of cellular lipids in maintaining HSCs in quiescent state[11, 12]. Recovery of lipogenesis and elevation of cellular lipid content in HSCs might be a strategy for attenuating HSCs activation and inhibiting fibrogenesis[13, 14]. Freshly isolated HSCs were gradually and spontaneously activated in the culture within 7 days[15], and the process was similar to the test procedure *in vivo*, providing a good model for explaining the potential mechanism of HSCs activation and the underlying therapeutic intervention to study the

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process[16, 17].

LDs are present in almost all normal cells, and contain a neutral lipid hub surrounded by a phospholipid monolayer in which amphiphilic proteins insert[18, 19]. One major member of these coating proteins is perilipins (plins), which are defined by N-terminal sequence similarity within and across species and quantitatively represent the most abundant signature of the LD machinery[20, 21]. The mammalian genome encodes five perilipin (Plin) genes with individual tissue-dependent expression patterns. Plin1 is expressed in white adipose tissue (WAT), brown adipose tissue (BAT), and steroidogenic tissue; Plin2 (Adipophilin) and Plin3 (TIP47) are ubiquitously expressed; Plin4 (S3-12) is highly expressed in adipocytes; Plin5 (LSDP5, OXPAT, MLDP, PAT1) is a newly discovered member in the Plin family and mainly expressed in oxidative tissues, including heart, skeletal muscle, and liver[22]. These proteins are crucial for the formation of cellular LDs and modulation of lipid homeostasis by regulating the arrival and consumption of intracellular lipids in the physiological settings[23–26]. However, any change of plins gene expression, knockout or over-expression, is recognized to accelerate the pathologic processes of many metabolic disorders, including fatty liver and insulin resistance[27–29]. For example, Plin5 knockout mice were susceptible to hepatic lipotoxicity induced by high fat diet (HFD)[13].

Previous study revealed that Plin1, 2, 3 and 4 were hardly detectable or did not change in quiescent HSCs or activated HSCs[30]. Recent work demonstrated that the expression of exogenous Plin5 significantly increased the levels of cellular lipid content and restored the formation of LDs in HSCs, as well as attenuated HSCs activation through inhibiting alpha-smooth muscle actin (α-SMA) gene expression and production of ECM[30], suggesting that upregulation of Plin5 expression might be a strategy for attenuating HSCs activation and inhibiting fibrogenesis.

Curcumin is a natural polyphenolic compound derived from turmeric, which has been found to have a lot of functions such as antioxidant, anti-inflammatory, antiproliferative, and proapoptotic activities[31]. Recent *in vitro* and *in vivo* studies have shown that curcumin could be an effective agent in inhibition of HSCs activation, including inducing gene expression of endogenousperoxisome proliferator-activated receptor-gamma (PPARγ) and adenosine monophosphate-activated protein kinase (AMPK), suppressing gene expression of αI(I) collagen, α-SMA, and protecting the liver from CCl4-caused fibrogenesis[32–35]. Besides, curcumin has been proven to be non-toxic even at high doses[36, 37]. Therefore, curcumin is a remarkable candidate for therapeutic strategies for liver fibrosis.

However, whether curcumin inhibition of HSCs activation is associated with LD formation is not fully understood. Furthermore, given that curcumin restores the LDs in HSCs, whether it also potentiates lipid accumulation in hepatocytes is another concern, the latter means the high risk of steatosis development. The current study aims at evaluating the effect of curcumin on lipid metabolism in HSCs and hepatocytes and further elucidating the underlying mechanisms. Results in this report provide evidence to support our initial hypothesis that curcumin restores the LD formation in activated HSCs, but not in hepatocytes, by stimulating Plin5 gene expression, which is closely associated with increased PPARγ and AMPK activities.

1 MATERIALS AND METHODS

1.1 Materials

Curcumin (purity >94%), Compound C [Compd C, a selective inhibitor for 5′-AMP activated protein kinase (AMPK)], and PD68235 (a specific PPARγ antagonist) were purchased from Sigma-Aldrich (USA). Curcumin was dissolved in 100% ethanol with a stocking concentration of 100 mmol/L. Antibodies used in this study were purchased from Santa Cruz Biotechnology (USA), except specific indications.

1.2 HSCs Isolation and Culture

Mouse HSCs were isolated by the pronase-collagenase perfusion *in situ* before density gradient centrifugation, as described previously[13]. Briefly, freshly isolated HSCs were used for RNA preparation, or cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). The cells were passaged in DMEM with 10% FBS. If there's no specific indication, semi-confluent HSCs with four to nine passages were used in experiments.

1.3 Western Blotting Analyses

Proteins from whole cell extracts were separated by 10% Tris-HCl SDS-PAGE and transferred to a PVDF membrane. Target proteins were detected by primary antibodies and subsequently by horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized by using a chemiluminescence reagent. β-actin was used as the internal invariable control for equal loading.

1.4 RNA Extraction and Real-Time PCR

Total RNA was extracted from the cells by the TRI-Reagent, following the protocol recommended by the manufacturer (Sigma, USA). Total RNA was treated with DNase I before the synthesis of the first strand of cDNA. Real-time PCR was performed using SYBR Green Supermix (Sigma, USA). The reactions started at 95°C and continued for 7 min, followed by 40 cycles of 95°C for 20 s, 54°C for 30 s and 72°C for 30 s. Data were analyzed using the LightCycler Software 1.5 (Roche Diagnostics GmbH, Germany). The threshold cycle value (Ct) was determined using

the following equation: ΔCt=Ct (target gene)–Ct (GAPDH). The mRNA expression of target genes is shown as a log ratio relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (log_2 –(ΔCt)). The following primers were used in this study:
abdh5: (F) 5′-TCTTGCTTGGACACAACCTG-3′,
(R) 5′-GAGGTGACTAACCCTTGATGG-3′;
ATGL: (F) 5′-AACACCAGCATCCAGTTCAA-3′,
(R) 5′-GGTTCAGTAGGCCATTCCTC-3′;
FAS: (F) 5′-CTGTTGGAAGTCAGCTATGAAG-3′,
(R) 5′-AGCTGGAGGAGCAGGCTGTG-3′;
Plin1: (F) 5′-CATGTCCCTATCCGATGCCC-3′,
(R) 5′-GGGAAGCGGCACATAGTGTA-3′;
Plin5: (F) 5′-CCATATGGACCAGAGAGGTGAAGA CACCAC-3′,
(R) 5′-GGAAGATCTTCAGGAGTCCAGCTC TGGCA-3′;
PPAR-γ: (F) 5′-ATGCCAAAAATATCCCTGGTTTC-3′,
(R) 5′-GGAGGCCAGCATGGTGTAGA-3′;
SREBP-1c: (F) 5′-TGCCCTAAGGGTCAAAACCA-3′,
(R) 5′-TGGCGGGCACTACTTAGGAA-3′;
Prkaa1: (F) 5′-GATCGGCCACTACATCCTGG-3′,
(R) 5′-GATGTGAGGGTGCCTGAACA-3′;
GAPDH: (F) 5′-CTACACTGAGGACCAGGTTGTCT-3′,
(R) 5′-GGTCTGGGATGGAAATTGTG-3′.

1.5 Detection of Intracellular Triglyceride (TG) and Free Fatty Acid (FFA) Levels

The lipids in treated cells were extracted with 5% NP-40 in water for TG detection or 1% Triton X-100 in pure chloroform for FFA detection. The TG or FFA contents were determined by Triglyceride or Free Fatty Acid Quantification Kit from BioVision, USA (Cat: K614-100, or K612-100, respectively) following the protocol recommended by the manufacturer. Protein concentration was quantitated by BCA assay (Pierce). The final lipid levels were expressed as pmol/μg protein.

1.6 Oil Red O Staining

HSCs were seeded on autoclaved cover slips in a 6-well plate and cultured in DMEM with 10% FBS for indicated days before fixation with 4% paraformaldehyde (30 min). LD was stained, as described previously[16].

1.7 Adeno-Plin5 Transduction

Adenovirus expressing mouse Plin5 (Ad-Plin5) and lacZ (Ad-LacZ) were from Vector BioLabs (USA). HSCs were transduced with 20 plaque-forming units (pfu) per cell of Ad-Plin5 or Ad-LacZ for 1 h in serum-free DMEM and cultured in 10% FBS DMEM for 48 h at 37°C in 5% CO_2 before oil red O staining and intracellular TG or FFA level detection.

1.8 Plin5 siRNA Transfection

Small interfering RNA (siRNA) targeting mouse Plin5 (sense GCCAC UCGCC UAUGA ACACU CUUU, antisense AAAGA GUGUU CAUAG GCGAG UGGC), and negative control siRNA (sense: CUCCG A AUUA GUCGC ACUCU CUCA, antisense: UGAG A GAGUG CGACU AAUUC GGAG) were designed by BLOCK-iT™ RNAi Designer and transfected into HSCs using oligofectamin (Invitrogen, USA). Briefly, a siRNA stock was diluted into 200 nmol/L with serum-free DMEM containing oligofectamin and incubated for 20 min at room temperature, prior to applying to each dish and cultured for 4 days. Culture media containing siRNA solution were changed every 2 days. The transfected cells were used for the next experiments.

1.9 Immuno-fluorescent Staining

The experiments were conducted as we previously described[30] using goat polyclonal primary antibody against Plin5 (1:50, sc-240627, Santa Cruz, USA) and donkey anti-goat IgG secondary antibody conjugated with Alexa Fluor 488 (1:300, cat# ab150129, Abcam, USA). Nuclei were stained by a mounting solution with DAPI. The slides were observed under a fluorescent microscope (Leica DM 4000 B, Germany). Representative views were presented.

1.10 Statistical Analyses

Differences between means were evaluated using an unpaired two-sided Student's *t*-test ($P<0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with controls were analyzed by ANOVA with the Dunnett's test for post hoc analysis.

2 RESULTS

2.1 Curcumin Dose-dependently Recovered LD Formation and Lipid Contents in Passaged Mice HSCs, but not in Hepatocyte Lines AML12

Previous studies have demonstrated that curcumin inhibits HSCs activation induced by various stimuli[16]. Whether the inhibition of curcumin is through increasing LD formation remains obscure. To evaluate the effect of curcumin on lipid metabolism in HSCs, the passaged mice HSCs and hepatocyte lines AML12 were treated with different doses of curcumin for 24 h prior to incubation with 100 nmol/L Palmitic acid for another 24 h. Intracellular LD formation was detected by oil red O staining. As shown in fig. 1A, compared to control HSCs, HSCs treated with curcumin showed apparent LD in a dose-dependent manner. However, no LD was detected in AML12 cells not only in control but also in the cells with curcumin treatments. These results indicated curcumin recovered LD formation in activated HSCs and unaffected hepatocyte lipid accumulation. Next, the intracellular lipid contents, including TG and FFA, were analyzed in HSCs. HSCs were treated with 20 μmol/L curcumin for different time lengths (0 to 72 h), and the data in fig. 1B showed that lipid contents were increased time-dependently in the first 24 h, but maintained in a steady state from 24

to 96 h, indicating 24 h is sufficient to saturate the lipid accumulation in cells. In the other hand, dose-effect analysis was performed in passaged HSCs treated with different concentrations of curcumin for 24 h. As expected, curcumin dose-dependently increased the TG (fig. 1C) and FFA (fig. 1D) levels. Compared to the control cells, TG level was increased by two folds and FFA level increased by three folds in the cells treated with 20 μmol/L curcumin. Taken together, curcumin recovered the lipid accumulation in activated HSCs and did not increase lipid contents in hepatocytes, suggesting that curcumin may inhibit HSCs activation without increasing the risk of steatosis developments.

2.2 Curcumin Regulated Expression of Genes Related to Lipogenesis and Lipolysis in Activated HSCs

The lipid accumulation inside cells largely depends on the imbalance of lipid metabolisms which is most characterized by the increased ratio of production to degradation. To evaluate the mechanism of curcumin in restoring the LD formation in activated HSCs, the effect of curcumin on the expression of genes related to lipogenesis (SREBP1c, FAS) and lipolysis (ATGTL, Abhd5) was observed. Passaged HSCs were treated with different doses of curcumin for 24 h, and the gene expression was assessed by Western blotting (fig. 2A) and real-time PCR (fig. 2B). It was revealed that curcumin dose-dependently increased SREBP1c and FAS levels while inhibiting ATGL levels, indicating that curcumin promoted lipid synthesis and suppressed lipolysis. Surprisingly, there was no significant difference in Abhd5 gene expression upon curcumin treatment.

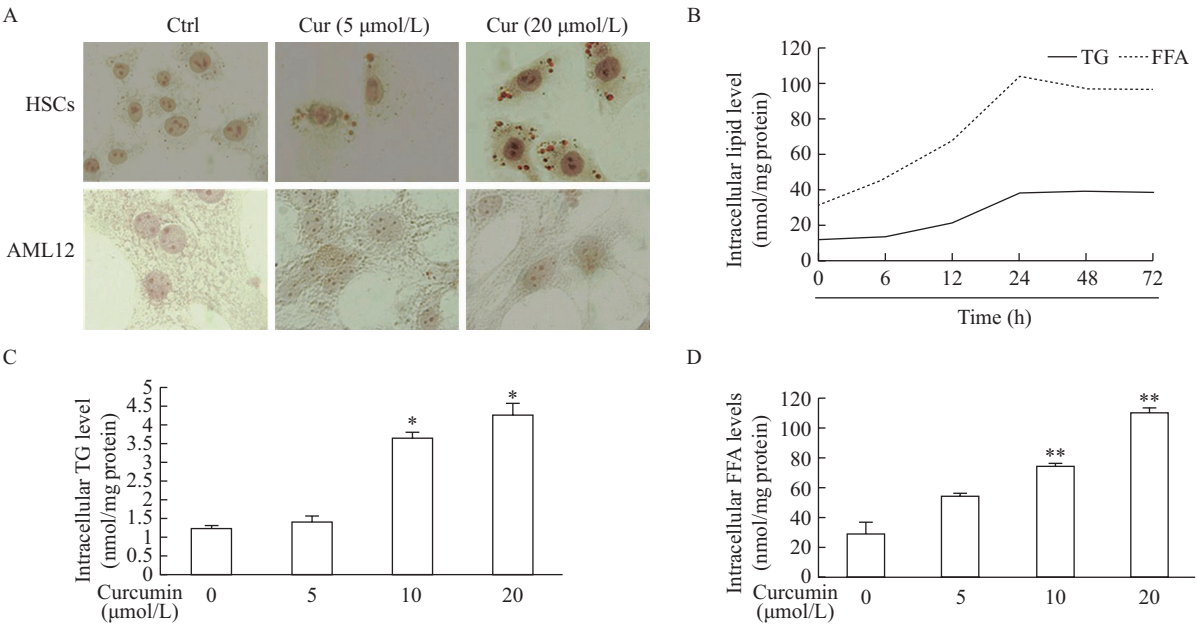

Fig. 1 Curcumin dose-dependently recovered lipid droplets formation and increased lipid contents in passaged mice HSCs, but not in hepatocyte lines AML12

A: intracellular LD in HSCs with oil red O staining (red circular particles), but not found in AML12 (×400); B: intracellular TG and FFA levels in HSCs treated with 20 μmol/L curcumin for different time lengths ($n \geq 6$); C and D: Intracellular TG and FFA levels in HSCs treated with various doses of curcumin for 24 h ($n \geq 6$). $^{*}P<0.05$, $^{**}P<0.01$ *vs.* cells without curcumin treatment. Ctrl: control; Cur: curcumin

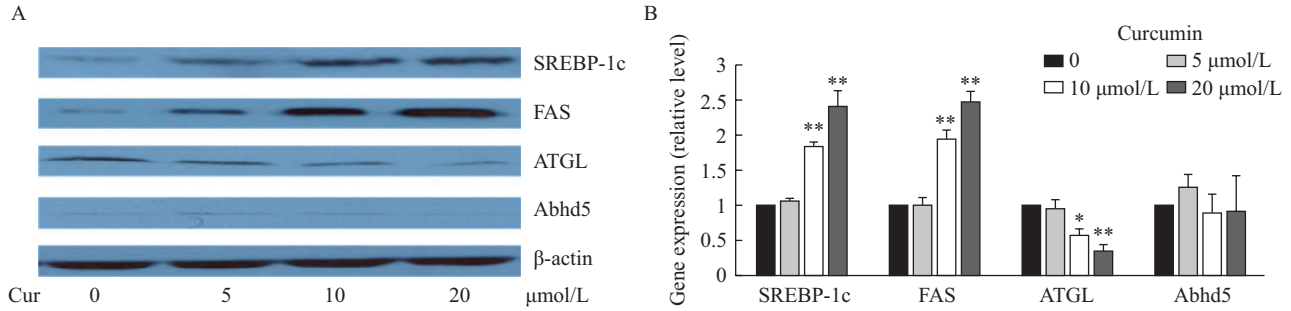

Fig. 2 Curcumin (Cur) regulated the expression of genes related to lipogenesis and lipolysis in activated HSCs

A: The expression of protein related to lipid metabolisms in HSCs ($n=6$); B: The mRNA levels of gene related to lipid metabolism in HSCs ($n=6$). $^{*}P<0.05$, $^{**}P<0.01$ *vs.* cells without curcumin treatment

2.3 Curcumin Elevated Plin5 Gene Expression in Activated HSCs

Prior study suggested that Plin5 plays a role as a lipolytic barriers on the LD surface, and its overexpression restores LD formation in activated HSCs[30]. We next investigated whether the alteration of lipid metabolism by curcumin was through regulating Plin5 expression. Passaged HSCs were treated with different doses of curcumin for 24 h, and Plin1 and Plin5 gene expression was detected by real-time PCR and Western blotting. Importantly, we found that curcumin dose-dependently increased Plin5 mRNA (fig. 3A) and protein levels (fig. 3B). However, there was no significant difference in Plin1 gene expression both in mRNA and protein levels between the cells with and without curcumin treatment. In addition, immunofluorescent analysis in fig. 3C further confirmed that curcumin recovered Plin5 gene expression which had been inhibited in activated HSCs. These observations indicated that curcumin selectively induced Plin5 expression, thus allowing increased storage of lipid, facilitated and stabilized the formation of LD.

2.4 Exogenous Plin5 Expression Had the Same Effects on LD and Lipid Levels in Activated HSCs as Curcumin Did

To further examine the effect of Plin5 on lipid accumulation in activated HSCs, activated HSCs were transfected with 20 pfu per cell of adeno-Plin5 or adeno-Lacz for 1 h, then cultured in regular medium for 48 h. Oil red O staining showed in fig. 4A that treated cells had significant increase in LD particles as compared with adeno-Lacz treated or control cells. In addition, intracellular TG (fig. 4B) and FFA (fig. 4C) levels were markedly elevated in adeno-Plin5 treated cells, but no significant difference was found in cells treated with adeno-lacz treatment compared with control cells. These data indicated that exogenous Plin5 expression promoted lipid accumulation in activated HSCs, which was similar to curcumin.

2.5 Blockade of Plin5 Attenuated Curcumin's Effect on Intracellular LD Levels and Lipid Levels in Activated HSCs

Next, 200 nmol/L Plin5 siRNA or ctrl siRNA were used to transfect passaged HSCs for the investigation of the role of Plin5 in curcumin inducing lipid accumulation. Fig. 5A showed that LD significantly increased in activated HSCs upon treatment with curcumin or curcumin plus ctrl siRNA. However, in Plin5 siRNA transfected cells, curcumin did not induce LD formation, indicating Plin5 silence abolished the restoration of LD by curcumin. Next experiments confirmed that Plin5 siRNA suppressed curcumin induced increases of TG (fig. 5B) and FFA (fig. 5C) levels. Taken together, these data suggested that Plin5 is involved in curcumin-mediated lipid accumulation and its gene expression is required for curcumin exerting its effects on lipid metabolism in activated HSCs.

2.6 Blockade of Plin5 Attenuated Curcumin's Effect on the Expression of Genes Associated with Lipid Metabolism in Activated Mice HSC

For the analysis of the effect of Plin5 on the gene expression associated with lipid metabolism regulated by curcumin, passaged HSCs were transfected with or without 200 nmol/L Plin5 siRNA or ctrl siRNA for 24 h, followed by treatment with or without 20 μmol/L curcumin for another 24 h. Whole cell lysates and total RNA were prepared. As shown in fig. 6A and fig. 6B by Western blotting and real-time PCR assays, SREBP1c and FAS were markedly increased in the cells treated with curcumin or curcumin plus siRNA/Plin5 siRNA

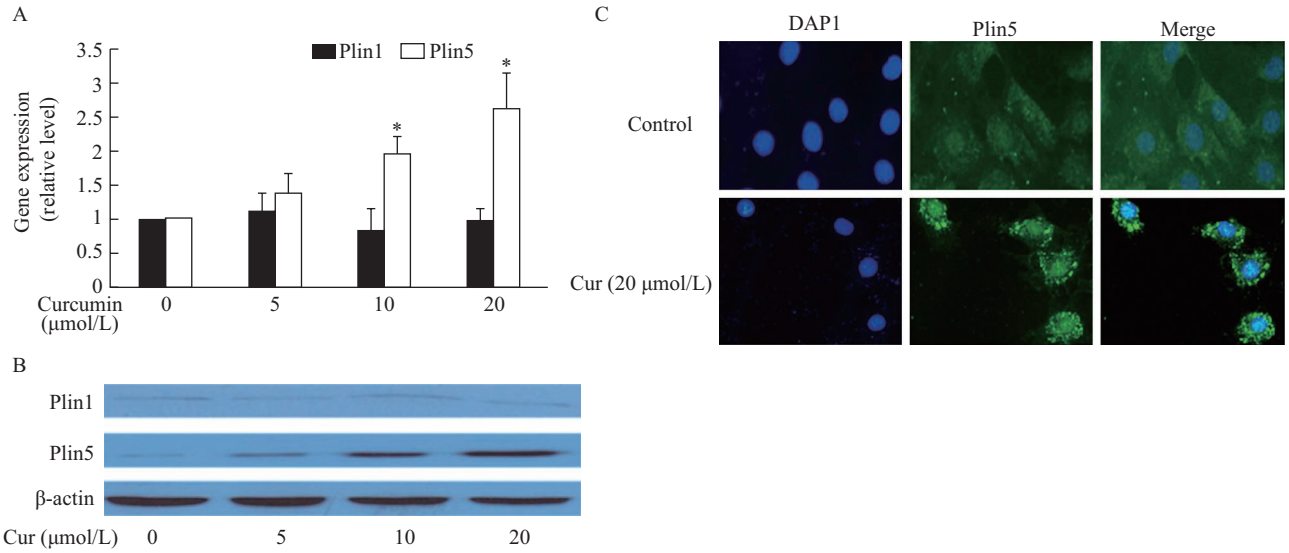

Fig. 3 Curcumin elevated Plin5, not Plin1 gene expression in activated HSCs

A: The mRNA levels of Plin5, Plin1 in HSCs treated with various concentrations of curcumin ($n \geq 6$), $^{*}P<0.05$ *vs.* cells without curcumin treatment. B: The protein levels of Plin5, Plin1 in HSCs treated with various concentrations of curcumin ($n=6$). C: Double staining of Plin5 and DAPI in HSCs treated or not treated with 20 μmol/L curcumin (×400). Cur: curcumin

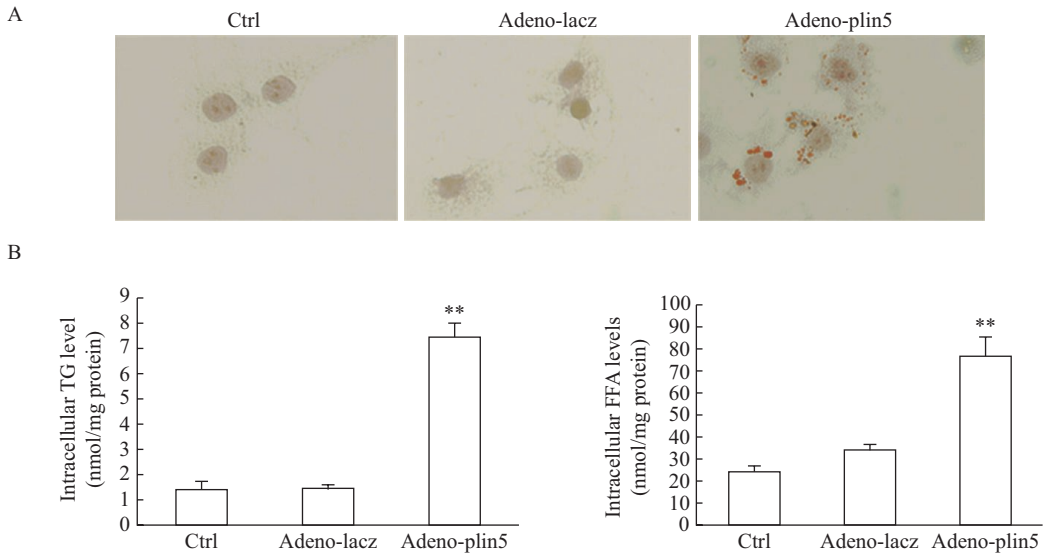

Fig. 4 Exogenous Plin5 expression had the same effects on lipid droplets and lipid levels in activated HSC as curcumin did
A: intracellular LD levels in HSC transfected with adeno-Plin5 or adeno-lacz. LD were indicated by red circular particles (×400). B and C: intracellular TG levels and FFA levels in HSCs, respectively (n=6). $^{**}P$<0.01 *vs.* control cells (Ctrl)

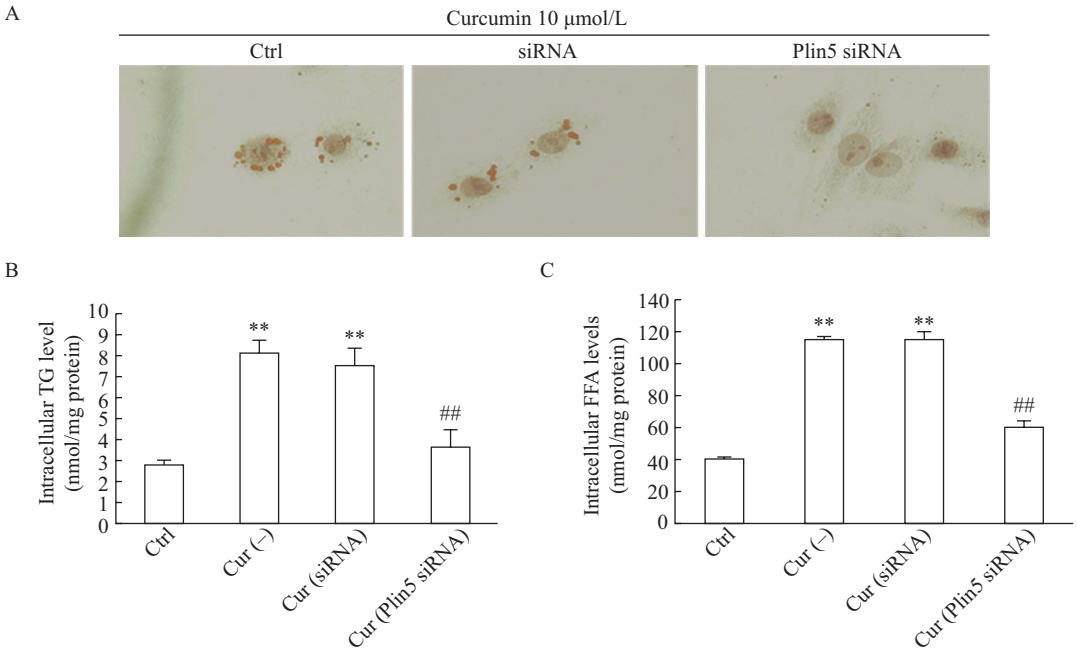

Fig. 5 Blockade of Plin5 attenuated curcumin's effect on intracellular LD levels and lipid levels in activated HSCs
A: intracellular LD levels in HSCs transfected with Plin5 siRNA or siRNA. LD was indicated by red circular particles (×400). B and C: intracellular TG levels and FFA levels in HSCs, respectively (n=6). $^{**}P$<0.01 *vs.* control cells; $^{\#\#}P$<0.01 *vs.* curcumin treated cells with siRNA transduction. Ctrl: control; Cur: curcumin; –: only treated with 10 μmol/L curcumin

transfection. In great contrast, HSCs treated with curcumin combined with Plin5 siRNA transfection showed significantly reduced levels of SREBP1c and FAS either in protein or in mRNA level. On the contrary, Plin5 siRNA transfection obviously reversed curcumin-inhibited ATGL gene expression, which was the representative of lipolysis. We also observed that there was no influence in Abhd5 gene expression among the cells treated with or without curcumin or combined with Plin5 siRNA transfection. These results collectively demonstrated that Plin5 gene expression was essential for the regulation of lipid metabolism induced by curcumin, resulting in lipid accumulation and LD formation in activated HSCs.

2.7 Inhibition of AMPK Activity Blocked Curcumin's Effect on Plin5 Gene Expression and LD Formation

Previous study verified that curcumin increased

AMPK activity[38] and AMPK activation was closely associated with Plin5 gene expression. To elucidate the mechanisms by which curcumin induced the expression of Plin5, we presumed AMPK activation by curcumin induced Plin5 gene expression, leading to the restoration of LD formation and the regulation of genes involved in lipid metabolisms and the elevation of cellular lipid content. To validate our presumption, passaged HSCs were treated with or without different doses of Compd C, AMPK specific antagonist, for 30 min prior to incubation with curcumin (20 μmol/L) for 24 h. As shown in fig. 7, Compd C dose-dependently reduced LD levels (fig. 7A), Plin5 protein level (fig. 7B) and mRNA level (fig. 7C), which were elevated by curcumin. Together, these results supported our hypothesis that curcumin induced AMPK activity, which promoted Plin5 gene expression and thus regulating lipid accumulation.

2.8 Inhibition of PPARγ Activity Eliminated Curcumin's Stimulatory Effect on Plin5 Expression and LD Formation

It has been verified that HSCs activation is coupled with dramatic down-regulated expression of PPARγ, and curcumin increased PPARγ activity in activated HSCs[13]. PPARγ is required for curcumin to inhibit HSCs proliferation and production of extracellular matrix. To investigate whether PPARγ also plays a role in curcumin's effect on Plin5 gene expression, passaged mouse HSCs were treated with 20 μmol/L of PD68235 (PD), PPARγ antagonist, for 30 min prior to incubation with curcumin (20 μmol/L) for 24 h. Data in fig. 8A showed that LD was not found in the cells treated with PD and curcumin, indicating PD blocked curcumin's stimulatory effect on LD formation. Further experiments demonstrated that curcumin-induced Plin5 gene expression in protein (fig. 8B) and

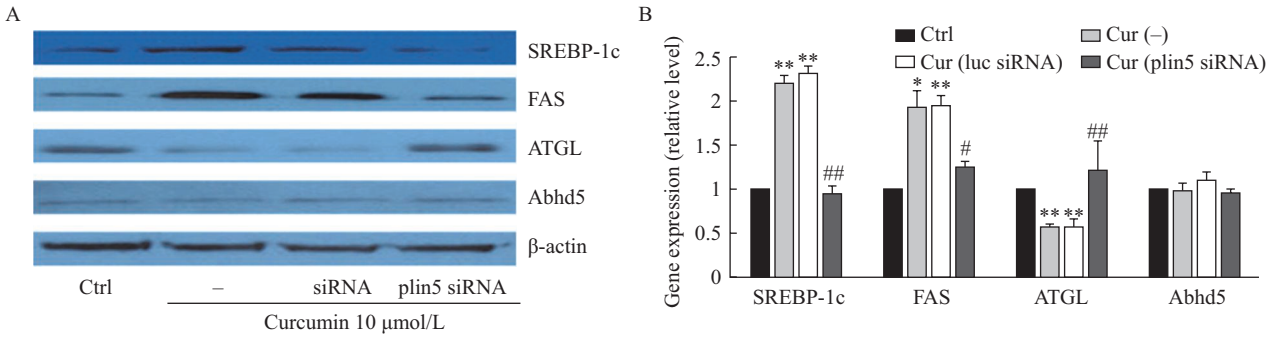

Fig. 6 Blockade of Plin5 attenuated curcumin's effect on the expression of genes associated with lipid metabolism in activated mice HSCs
A: the expression of protein related to lipid metabolisms in HSCs (n=6); B: the mRNA levels of gene related to lipid metabolism in HSCs (n=6). $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control cells (Ctrl); $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* cells treated with curcumin (Cur) only. –: only treated with 10 μmol/L curcumin

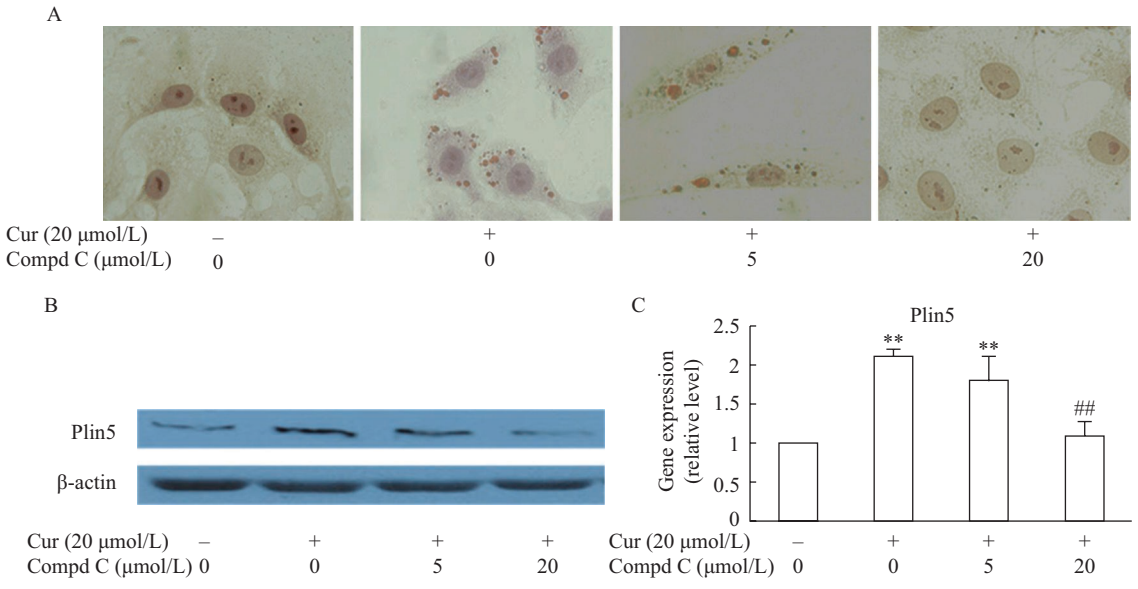

Fig. 7 Inhibition of AMPK activity blocked curcumin's effect on Plin5 gene expression and LD formation
A: intracellular LD levels in HSCs treated with various concentrations of Compd C and 20 μmol/L of curcumin. LD was indicated by red circular particles (×400). B and C: The protein levels and mRNA levels in HSCs treated with various concentrations of Compd C and 20 μmol/L of curcumin (n=6). $^{**}P$<0.01 *vs.* control cells; $^{\#\#}P$<0.01 *vs.* cells treated with curcumin (Cur) only

mRNA (fig. 8C) levels was significantly suppressed by pretreatment with PD in the cells. These results suggested that PPARγ was involved in curcumin-induced up-regulation of Plin5 gene expression in activated HSCs.

2.9 Relationship between PPARγ and AMPK in Curcumin's Effect

Our above results demonstrated that both AMPK and PPARγ participated in the effect of curcumin on Plin5 gene expression. To further understand the relationship between AMPK and PPARγ, we used the inhibitors against AMPK or PPARγ to pretreat cells for 30 min, then observed another molecule's gene expression. In the experiment about AMPK inhibition, Compd C was found to dose-dependently block PPARγ gene expression (fig. 9A and fig. 9B), PPARγ was inhibited approximately by up to 100% in the cells treated with 20 μmol/L Compd C. On the other hand, curcumin-stimulated phosphorylation of AMPK and gene expression of Prkaa1 (AMP-activated protein kinase alpha 1 catalytic subunit) were significantly inhibited by 20 μmol/L of PD (fig. 9C and fig. 9D, respectively). Together, these results revealed that PPARγ and AMPK had positive synergistic effects on curcumin stimulatory lipid accumulation in activated HSCs.

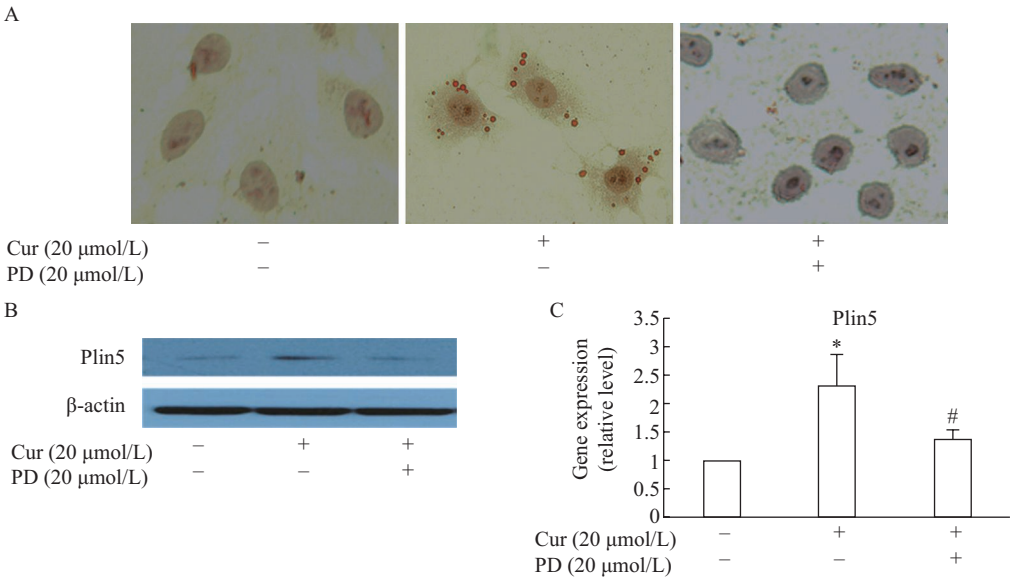

Fig. 8 Inhibition of PPARγ activity eliminated curcumin's stimulatory effect on Plin5 expression and LD formation
A: Effect of curcumin (Cur) and PD on intracellular LD levels in HSCs. LD was indicated by red circular particles (×400). B and C: Effect of Cur and PD on the protein levels and mRNA levels of Plin5 in HSCs (n=6). $^{*}P$<0.05 *vs.* control cells; $^{\#}P$<0.05 *vs.* cells treated with Cur only

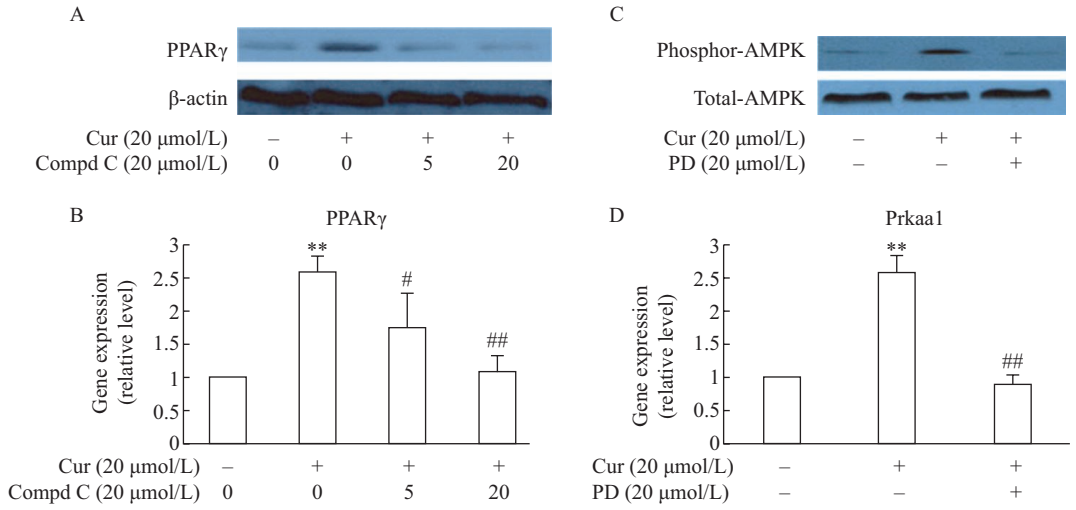

Fig. 9 The relationship between PPARγ and AMPK in curcumin's effect
The expression of PPARγ in protein levels (A) and mRNA levels (B) in HSCs treated with Compd C at indicated doses and curcumin (20 μmol/L) (n=6). The expression of AMPK in phosphorylated protein levels (C) and mRNA levels (D) in HSCs treated with Compd C at indicated doses and curcumin (20 μmol/L) (n=6). $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control cells; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* cells treated with curcumin (Cur) only

3 DISCUSSION

Our present study has identified that curcumin selectively recovers the lipid accumulation in activated HSCs, not in hepatocytes. Curcumin augments Plin5 gene expression, and the interruption of Plin5 expression eliminates curcumin's effect on LD formation and cellular lipid contents in activated HSCs. Previous study has shown that curcumin increases PPARγ and AMPK activities[34, 35]. This study has further found that the inhibition of PPARγ or AMPK activation significantly reduces curcumin-stimulated LD and Plin5 gene expression. In addition, PPARγ and AMPK had positive interaction, and their combined actions augment curcumin's effect in increasing Plin5 gene expression, which facilitates lipid biosynthesis more than consumption, resulting in LD formation and reversion of HSCs into quiescent state. Curcumin might be a promising agent for attenuating HSCs activation and inhibiting fibrogenesis, with the minor risk of steatosis development.

The regulation of lipid storage in liver should be prudent, since the accumulations of lipid in hepatocytes and HSCs lead to different outcomes. Under normal circumstances, liver plays an essential role in maintaining lipid homeostasis[38]. Hepatocytes are the primary site of lipid (mainly cholesterol and triglyceride) biosynthesis and storage as well as lipid excretion, converting lipid to bile acids and removing free lipid as lipid metabolites via biliary excretion[38, 39]; HSCs store as much as 50%–60% of the total retinoid in the entire body, being the main cellular site for vitamin A[41]. However, more than 5% of the extracellular lipid accumulation of liver cells leads to hepatic steatosis, which may develop into multiple dysfunctions such as alterations in beta-oxidation, very low density lipoprotein secretion, and pathways involved in the synthesis of fatty acids. In addition, an increased circulating pool of non-esterified fatty acid may also serve as a major determinant in the pathogenesis of fatty liver disease[42]. Previous studies have shown that exogenous Plin5 gene expression increases LD formation in HSCs[30], but there is a question suggesting that Plin5 may also affect lipid accumulation in hepatocytes, and if so, steatosis would be deteriorated by Plin5. Therefore, the best way against liver fibrogenesis is the strategy specifically targeting HSCs without affecting hepatocytes. Our present report, for the first time, proved that curcumin meets the criteria to selectively up-regulate LD formation in activated HSCs.

Neutral lipid accumulation and perilipin production are required for LD formation. De novo synthesis is one of the main sources for lipid accumulation, and several transcription factors and enzymes are involved in the process. SREBP-1c (also called adipocyte determination and differentiation-dependent factor 1) is a key transcription factor for the promotion of lipid synthesis and adipocyte differentiation in liver. The ratio of SREBP-1c to SREBP-1a mRNA is 9:1 in liver, and SREBP-1c is the predominant transcript in liver[43]. *In vivo* studies of hepatic SREBP-1c overexpression in transgenic mice showed that overexpression of SREBP-1c increased liver lipid accumulation and insulin resistance, whereas SREBP-1 knockout mice showed defects in induction of lipase by feeding[44]. The expression of SREBP-1c was increased in resting HSCs, and the recovery of this factor in activated HSCs induces a rapid reversal of the cell phenotype to resting HSCs. The level of SREBP-1 is associated with the expression of genes involved in fatty acid synthesis (FAS)[45]. In terms of perilipins, Plin5 is considered as the main molecule in the HSCs[30]. In this study, we demonstrated that curcumin dose-dependently increased the gene expression of SREBP1c and FAS as well as Plin5, indicating curcumin augments LD formation, at least in part, through stimulating triglyceride synthesis and inducing perilipin production in activated HSCs.

Besides playing an importance role in the formation, stability, and transport of LD in the cells, Plin5 is involved in the regulation of lipid metabolism[46, 47], through providing a scaffold for adipose triglyceride lipase (ATGL) and CGI-58 at the LD surface and promoting the colocalization and functional interaction of ATGL and α-β hydrolase domain-containing protein 5 (Abhd5) in the basal state[48]. ATGL is the rate-limiting lipase that elicits lipolysis by hydrolyzing the first ester bond and releasing the first fatty acid. Abhd5 is also the coactivator of ATGL[49]. The complete activation of ATGL is closely related to abhd5. Abhd5 in cytosol is directed to LDs by LD proteins. Interruption of their interactions could greatly affect the activity of ATGL. At the molecular level, Plin5 assumes the function of a scaffold for two participants, namely Abhd5 and ATGL. It has been indicated that the C-terminus of Plin5 acts as a binding domain of ATGL and Abhd5. ATGL and Abhd5 compete for Plin5 binding sites, once combined, ATGL is effective in inhibiting Abhd5 binding[50]. The interaction between Abhd5 and Plin5 is indispensable for regulating the activity of ATGL at LD, and the process is dynamic. Therefore, the coordination of the triangle relationship among Plin5, ATGL and Abhd5 is critical for LD metabolism. In this study, curcumin inhibits ATGL gene expression while having no influence on Abhd5 level, suggesting that curcumin might reduce the opportunity of ATGL interacting with Abhd5; in addition, elevated Plin5 level by curcumin stimulation facilitates Plin5-Abhd5 interaction. The data support that curcumin may exert its effect through the two aspects together to limit ATGL activity, and thus inhibiting lipolysis in LD. Additional experiments are ongoing to further explore the impact

of curcumin on the protein-protein interaction between Plin5 and ATGL or Abhd5, and its role in the reduction of lipolysis in HSCs.

AMPK, a nutrient sensor and an anti-lipolytic factor, is another key regulator of intracellular lipid metabolism. AMPK plays a role in lipid regulation by phosphorylating and inactivating HSL and blocking its translocation to LD[50]. Previous study showed that exogenous Plin5 expression induced the activity of AMPK; inhibiting AMPK activity by its inhibitor Compd C attenuated the roles of Plin5 in elevating cellular lipids and in restoring the formation of LD in HSCs. Furthermore, AMPK activation by AICAR was found to be directly linked with the inhibition of HSCs[30]. There is ample evidence that AMPK regulates the proliferation and myofibrogenic activation of HSC through a variety of signaling mechanisms. Recent studies showed that AMPK activation significantly weakens TGF-β1 functions, and the induction of α-SMA by TGF-β1 treatment was reversed by AMPK activator in HSC-T6 cells[51]. Many compounds are known as AMPK activators through an indirect manner, via increasing the ratio of intracellular AMP to ATP or via activation or upregulation of upstream kinases[52]. In this study, we found that curcumin promoted AMPK activity, and interruption of AMPK activity significantly diminished curcumin's effect on Plin5 gene expression in activated HSCs, indicating that AMPK activation is the important step for curcumin to exert stimulatory effect on PD formation. The underlying mechanism remains incompletely understood, the possible explanation is the activation of AMPK promotes the phosphorylation of the transcription factor Nrf2, which triggers the translocation of Nrf2 from the cytoplasm into the nucleus[53], leading to its binding to ARE and the induction of Plin5 expression. In addition, exogenous Plin5 expression induced AMPK activity in activated HSCs[30], these positive reciprocal interactions may further amplify the activity of curcumin.

PPAR-γ is a nuclear transcription factor regulating fatty acid metabolism. It has been proven that PPAR-γ activation is the prerequisite for curcumin inhibiting HSCs activation[54, 55]. In this study, PPARγ antagonist markedly diminished curcumin's effect on Plin5 gene expression, that means, PPARγ also participates in the process of curcumin-induced LD formation. However, the relationship between AMPK and PPARγ is complicated. One of the action patterns is ATP-dependent AMPK-PgC-Ppar pathway, in that its impairment can lead to obesity, giving rise to disease[57, 58]. Here, we found AMPK and PPARγ had the mutual stimulatory effects. Emerging evidence has identified that AMPK-PPAR pathway mediates cell apoptosis and inhibits cell proliferation, especially in cancer cells, suggesting the possibility of utilizing the pathway as a therapeutic target in apoptosis-resistant cancers[59] and diabetes[60]. Since curcumin has the ability to increase both AMPK and PPARγ activities, it may potentially become the candidate to treat cancers and diabetes, as well as liver fibrosis.

To rule out the influence of curcumin, as a yellow products, in some assay, we did preliminary dose-effect tests. Our tests have shown that when the concentration of curcumin reaches 30 μmol/L or over, the color will interfere our assays, such as lipid contents (data not shown). To overcome this interruption, our present study chose 20 μmol/L of curcumin as the maximal treating dose.

In summary, the effect of curcumin on lipid levels in activated HSC, is shown in fig. 10. Curcumin induces Plin5 gene expression through AMPK-PPARγ pathway in HSCs on activation. The increased Plin5 expression in HSCs promotes lipogenesis and inhibits lipolysis. These effects collectively recovers the formation of cellular LD, and reverses HSCs into quiescent state, as a consequence, liver fibrogenesis is inhibited by curcumin. It is emphasized that our findings do not exclude the other effects and mechanisms of curcumin in the formation of LD in activated HSCs. The experiments about the role of curcumin in lipid metabolism *in vivo* are being explored.

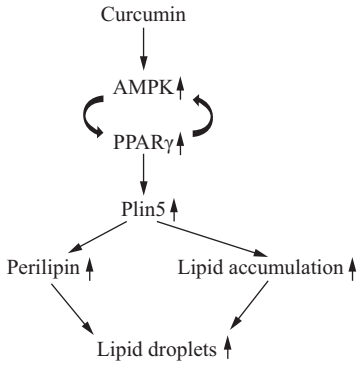

Fig. 10 Schema of the underlying mechanism of curcumin in the promotion of LD formation in HSCs
Curcumin restores LD formation by stimulating Plin5 gene expression through promoting AMPK and PPARγ activities, leading to increased perilipin and intracellular lipid accumulation, which contributes to the inhibition of HSCs activation

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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